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Research Article

Experimental Evidence for Neural Progenitor Cells in Adult Substantia Nigra

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Abstract

The substantia ("black substance" in Latin) is a long nucleus located in the midbrain but considered functionally a part of the basal ganglia because of its reciprocal connections with other brainstem nucleic. Degeneration of the pars compacta of the substantia nigra result in reduction of the availability of the neurotransmitter dopamine. This lack of dopaminergic innervation to the stratum results in disorders associated with hypokinesia or reduced motor movement. However, Parkinson disease is a result of reduced functioning substantia nigra. A few studies have shown that progenitor cells present in different areas of the adult central nervous system (CNS) but specificity (i.e. SNc) whether such cells reside in the adult SNc and whether they have the potential to replace degenerating neurons effects is unknown. The purpose was to investigate a population of actively dividing progenitor cells in the adult SN, after removal from the SN, these progenitor cells immediately have the potential to differentiate into neurons. Transplantation of freshly isolated SN progenitor cells into the adult hippocampus showed that these cells also have a neuronal potential under *in vivo* conditions. These results suggest that progenitor cells reside in the adult SN and can give rise to new neurons when exposed to appropriate environmental signals.

Keywords: Parkinson's Disease, Neural Progenitor Cells, Cell Replacement, Gliogenesis, Transplantation, Substantia Nigra

Introduction

Substantia consists of two components, the pars compacta and the pars reticulate, which have different connections and uses different neurotransmitters. Degeneration of the pars compacta of the substantia nigra result in reduction of the availability of the neurotransmitter dopamine [1]. This lack of dopaminergic innervation to the stratum results in disorders associated with hypokinesia or reduced motor movement. Parkinson's disease (PD), progressive loss of dopaminergic neurons in the substantia nigra pars compacta (SN) leads to debilitating motor dysfunction. The International Parkinson and movement disorder society established treatment with typical antiparkinson medication levodopa (L-DOPA) with dopamine agonist being used as replacement for amelioration of motor deficits. Olanow and Colleagues shows experimental therapies to restore dopaminergic neurotransmission by exogenous cell replacement, which is achieved by transplanting fetal dopaminergic neurons into the striatum, the main target area of the SN. However, the availability of cells, paucity of dopaminergic neurons in grafts, immunological response has hindered the success record of transplantation strategies after Transplantation, probable failure of cells to integrate into the host circuit, and the low percentage of survival of grafted cells [1]. Limited study of regenerative capacity of the adult CNS is and has been considered to be postmitotic incidence. However, growing evidence from Altman and his colleaques shows new mature neural cells are generated throughout adulthood, suggesting that the adult CNS retains the ability for endogenous cell replacement. Kuhn., *et al*. emphasized the cell derivatives from actively dividing progenitor cells, which display a broad or restricted differentiation pattern depending on their site of residence. Gage., *et al*. reiterate the generation of mature cells of all neural lineages, including neurons, has been consistently demonstrated in only two distinct areas of the forebrain In

contrast, in the adult spinal cord progenitor cells give rise to glial cells only. However, vivo differentiation pattern may not reflect the entire lineage potential of resident progenitor cells, because regional environmental cues appear to restricting situ differentiation to distinct neural lineages. For example, *in vitro* analysis and transplantation studies have which suggests that cortical progenitors in fact have a broader differentiation potential than observed under normal conditions and adult progenitor cells from the spinal cord have the potential to give rise to cells of all neural lineages according to Weiss and Colleagues. But, till now it is unclear whether cells with the potential to give rise to new neural cells exist in the adult SN and whether active cell replacement occurs in this CNS region. In this study, we investigate the neural progenitor cells persist throughout adulthood substantia nigra. Our results indicate that cells with a broad differentiation potential, which includes neurons, astrocytes, and oligodendrocytes, are present in the adult SN.

Methodology

In vivo labeling techniques

Young adult male Fisher 72 mice were housed in standard cages (10-11 weeks of age) was used. Mice were housed in standard cages and had an access to food and water. Animals were given retrovirus injections (with green fluorescent protein). 14 mouse were deeply anesthetized and injected stereotactically with 3.5 µl green fluorescent protein (GFP) retrovirus (10 × 105 IU/µl) into the left SN; mediolateral region, -4.2; anteroposterior, -7.4; dorsoventral (DV), -7.7 from bregma; nose piece, -4.3. Animals were anesthetized and perfused intracardially with 6% methyl formaldehyde in 110 mm phosphate buffer, pH 7.3 in 36 hr later. Bromomonoxyuridine injections. For birth dating studies, thirteen animals received a single intraperitoneal injection of bromodioxyuridine (BrdU) (63 mg/kg). Animals were perfused at 1.5 hr or 2 d after injection. For phenotype studies, 13 animals received intraperitoneal injections of BrmU (100 mg/kg) each day for 10 d. At 1 d after injection, one-half of the animals (n = 10) were perfused. The remaining animals (n = 10) were perfused at 4 weeks after injection. 6-Hydroxydopamine lesions.

Fourteen animals were injected stereotactically with two deposits of 1.5 µl of 6-hydroxydopamine (6-OHDA) (Sigma) (3 µg/µl in 0.6% NaCl supplemented with 0.04% ascorbic acid) into the left medial forebrain bundle (AP, -3.8; ML, -1.5; DV, -7.3 and -7.1 from bregma; nose piece, -3.3). Twelve animals received injections of vehicle. One day after injury, animals received intraperitoneal BrdU injections (100 mg/kg) each day for 12 consecutive days. Animals were perfused 4 weeks after the last BrdU injection.

Isolation and cell culture

Adult progenitor cells were isolated as described previously (Palmer., *et al*. 1999). The SN was dissected using a dissection microscope. Contamination by ependymal and subependymal cells was avoided by complete removal of tissue adjacent to the ventricle. The pial surfaces were trimmed from the tissue to remove the meninges. Tissues were digested in papain of 2.5 U/ml, DNase (250 U/ml) and neutral protease of 1 U/ml Dispase; Boehringer dissolved in HBSS. Whole digested tissue was washed and suspended in DMEM-10% fetal bovine serum (FBS). An equal volume of Percoll solution consisting of nine parts of Percoll and one part of 10× PBS was added. The cell suspension was centrifuged for 30 minutes, 18°C, at 20,000 ×g. Cells from the low-buoyancy fraction were harvested, rinsed in PBS, and plated onto Porn/Lam-coated tissue culture dishes in DMEM-10% FBS. The medium was replaced after 16 hr with serum-free growth medium consisting of DMEM/F-12 (1:1) supplemented with N_2 supplement Growth factors were added at the following concentrations: human FGF2 20 ng/ml, MN, 40 mg/ml. To promote differentiation, growth medium was replaced with DMEM/F-12 containing 2% FBS and 10µm of all-trans retinoic acid. For immediate differentiation experiments, two additional differentiation paradigms were applied: 2% FBS and 10 µm of forskolin according to Palmer and colleaques. Progenitors were cultured for 10 days in growth medium, harvested with trypsin-EDTA solution, washed one time with DMEM/F-12, and suspended to a final concentration of 72 cells/ml in growth medium supplemented with 2 µg/ml polybrene. Volumes of NIT-GFP retrovirus sufficient to infect 10 - 20 cells were added to 0.2 ml of cells and then incubated for 30 minutes at 37°C. The cells were pelleted, resuspended in growth medium, and plated into 6 cm tissue culture dishes. Day one later, the locations of individual green cells were marked. Adjacent clones closer than 1 cm apart were excluded from the study. Cells were grown in growth medium for 7 d and then switched to differentiation medium for another 7d. Cells were fixed for 20 min with 6% paraformaldehyde. Immunofluorescent staining. Floating tissue sections or cells were rinsed with Tris-buffered saline (TBS) and then blocked for 30 min at room temperature in TBS containing 0.3% Triton X-100 and 5% preimmune donkey serum (TBS++).

Samples incubation

Samples incubated in TBS++ containing dilutions of primary antibodies for 24 - 72 hr at 4°C. Samples were washed three times with TBS for 10 minutes at room temperature and blocked in TBS++ for 1 hr. Samples were then incubated for 2 hr with secondary antibodies conjugated to aminomethylcoumarin, fluorescein isothiocyanate and cyanine. Secondary antibodies (donkey;

Jackson Immuno Research, West Grove, PA) were used at a final dilution of 1:250 in TBS++. Samples were washed three times with TBS, treated with 10 mg/ml 4′,6′diamidino-2-phenylindole (DAPI) (Sigma) for 10 min, and coverslipped in 20% polyvinylalcohol, Air Products and Chemicals, in 50% glycerol (w/v) containing 2.5% w/v 1,4-diazobicyclo-[2.2.2]-octane (Sigma).

BrdU standing procedure

Samples were pretreated with 40% formamide in 2× SSC for 1.5 hr at 65°C, followed by 15 minutes in 2× SSC, 30 min in 2N HCl at 37°C, 10 minutes in 0.1 m borate buffer, and six 15 min rinses in TBS, pH 7.5. Primary antibodies generated in mice, were used rats, mouse anti-adenomatous polyposis coli tumor suppressor gene, mouse anti-A2B5 (1:100; Boehringer Mannheim), and mouse anti-TH. Fluorescent samples were evaluated using a Bio-Rad imaging system. Quantification of newly generated cells. Estimation of the number of BrdU-positive cells located within the adult SN was achieved by using the optical fractionator sampling design and formula according to study of west 1999. Stereo Investigator version 2001 stated that every sixth 40 µm coronal section of the midbrain was evaluated after double-immunostaining for TH and BrdU with the aid of a Stereo Investigator software-controlled stage attached to an Olympus BH-2 fluorescence microscope and Dage MTI CCD-300TIFG video camera, the SN was delineated using the TH immunostaining as guide. Counts were made using an unbiased counting frame superimposed on the image of the SN viewed under a 20× objective (sampling frame area, 10,000 μm^2; sampling frame height, 26 µm; top and bottom guard zones, at least 3 µm; sampling intervals, x = 240 µm, y = 150 µm; This procedure were performed according to Palmer., *et al*. principle.

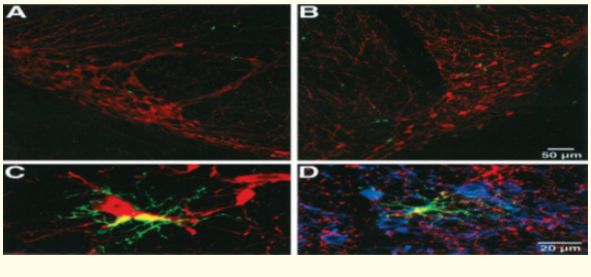

Figure 1

Proliferating cells are present in the adult SN. A, BrdU-labeled cells (green) are detected 2 hr after injection. B, at 3d after injection, more BrdUpositive cells (green) are present in the SN. Cells are mostly found in doublets. A, B, TH in red. C, D, Proliferating cells are also detected by injection of a GFP retrovirus into the SN. D, Some of the infected cells express NG2 (red, colabeling with GFP in yellow). TH in red (C) or colocalization with lineage specific markers. The number of transplanted cells that differentiated into a particular phenotype was expressed as a percentage of the BrdU-positive cells examined.

Analysis and Results

We investigate the phenotypical structure of proliferating cells and their lineage in the SN, 72 animals randomly selected, BrdU-positive cells per animal in this region were evaluated for colabeling with each phenotypic marker. For each BrmU-positive cell, the complete cell nucleus was followed through the z-axis, and only cells with a well circumscribed, immunopositive cell body or nucleus were considered positive for a particular phenotype. The labeling index was calculated by dividing the number of cells that were double labeled for BrdU and a phenotypic marker by the number of evaluated BrdU-positive cells. To determine the phenotype of cultured progenitor cells, 1000 cells per condition observed in nonoverlapping fields of view were evaluated for the expression of phenotypic markers.

Cell origin in SN

To determine whether the adult midbrain contains dividing cells, animals were pulsed with a single dose of BrdU and killed 4 hr or 5 days later. At 2 hr after injection, cells undergoing DNA replication had incorporated the label but had not had time to migrate far from the site of incorporation. At this time point, BrdU-positive cells were detected throughout the entire midbrain including the SN, indicating that proliferating cells are present in this region. At 5 days after injection, the number of BrdU-labeled cells in the SN appeared to have increased, and cells were found predominantly in doublets, suggesting that cells had divided locally. The presence of locally dividing cells in the adult SN was confirmed by stereotactic injection of a Moloney murine leukemia virus (MoMLV)-based GFP retrovirus into the SN. MoMLV-based retroviruses infect only dividing cells. At 36 hr after injection, GFP-positive cells were detected in the SN. These cells had small round cell bodies and elaborate processes and were in some cases colabeled for NG2, an early glial progenitor cell marker Proliferating cells are present in the adult SN. A, BrdU-labeled cells (green) are detected 2 hr after injection. B, At 3 d after injection, more BrdU-positive cells (green) are present in the SN. Cells are mostly found in double.

Phenotypic analysis of newborn cells in the adult SN

To determine the phenotype and the fate of dividing cells, animals were injected daily with BrdU for 10d and perfused at 1d and 4 weeks after injection. Stereological analysis showed that the num-

ber of BrdU-positive cells did not differ significantly between those time points [2761 ± 381 (average ± SEM) at 1 d after injection and 2943 ± 505 at 4 weeks after injection], suggesting that a significant proportion of dividing cells or their progeny was maintained during this period. The relative contribution of proliferation, survival, and cell death to the maintenance of newly generated cells was not assessed in this study [1].

Sections were stained with glia- and neuron-associated markers. One-half of the BrdU-positive cells in the SN co labeled with the potential glial progenitor marker NG2 at 1d after injection (49.6 ± 3%). At 4 weeks after injection, the percentage of NG2-labeled, BrdU-positive cells had decreased only slightly (42.6 ± 1.1%), Lendahl and colleagues suggesting that many cells remained as glial progenitor cells in the SN. At 1 d after injection but not at 4 weeks after injection, a small population of BrdU-positive cells (0.1%) stained for the intermediate filament nestin which is expressed by multipotent neural progenitor cells during development [1]. These BrdU/nestin-positive cells were not associated with blood vessels, indicating that these cells were neural progenitor cells and not endothelial cells, which have been described to express nestin in the adult CNS [2]. Few newborn cells (<0.1%) expressed mature glial markers (S100β) at the early time point. In contrast, at 4 weeks after injection, 14% of BrdU-labeled cells had differentiated into oligodendrocytes (APC+/GFAP-) (12.9 ± 0.7%) or astrocytes (S100β+) (1.4 ± 0.2%) (Figure 3a and 3b). Very few BrdU-positive cells (<0.1%) expressed Ox42 at both time points, indicating that few newborn cells in the SN are microglia. Phenotype of BrdU-positive cells in the SN after a 10d BrdU pulse. A, One-half of the BrdU-positive cells (green) express the glial progenitor marker NG2 (blue). B, Some BrdU-positive cells (blue) that are not associated with blood vessels express. Progenitor cells in the SN differentiate into glia 4 weeks after the final BrdU injection. Colocalization of a BrdU-positive nucleus (blue) with the oligodendrocyte marker APC (green). B, Colocalization of a BrdU-positive nucleus (blue) with the astrocyte. To investigate the possibility that new neurons are generated in the SN, sections were stained with BrdU and β-tubulin III or NeuN. In addition, all sections were stained for TH to outline the SN. Hence, we were able to investigate the possibility of generation of new dopaminergic neurons throughout the entire SN. Multiple BrdU-positive nuclei nuclei per animal) seemed to be associated with TH-positive cell bodies or NeuN-positive nuclei at both time points. However, careful analysis by confocal z-series of each of these cells revealed that BrdU-labeled nuclei belonged to cells that were in close proximity to the nuclei of NeuN-positive or TH-positive neurons. No newly generated neurons were found in animals that were killed at earlier time points (1 and 2 weeks after injection), excluding the possibility that progenitor cells had differentiated into neurons that failed to survive (data not shown). In addition, no newborn neurons were observed in adjacent structures in the entire ventral midbrain at any time point. Therefore, no convincing evidence for *in vivo* neurogenesis in the SN or the ventral midbrain was found at any of the time points examined.

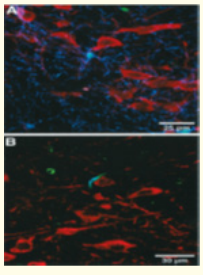

Figure 2

Phenotype of BrdU-positive cells in the SN after a 10 d BrdU pulse. A, One-half of the BrdU-positive cells (green) express the glial progenitor marker NG2 (blue). B, Some BrdU-positive cells (blue) that are not associated with blood vessels express the multipotent progenitor marker nestin (green). A, B, TH in shown in red.

Isolation of progenitor cells from the SN

Palmer hypothesized the Isolation of progenitor cells from the SN and investigates isolated cells from the SN using a protocol that enriches for progenitor cells from the adult brain. This procedure allows early *in vitro* analysis of progenitor cells and avoids prolonged culturing with exposure to high concentrations of growth factors, which can lead to transformation of cells and may dramatically alter their characteristics [1]. SN tissue for progenitor cell isolation was harvested by microdissection under a microscope. Although, this technique allowed complete removal of the nonadjacent ependymal and subependymal tissue, the possibility remains that the preparation was slightly contaminated with tissue from immediately adjacent tissue (e.g., ventral tegmental area) because of the proximity of structures in the ventral midbrain. To determine whether SN cells that were actively dividing *in vivo* were contained in the isolated cell population; animals were injected with high doses of BrdU (200 mg/kg) on 7 consecutive days before isolation. Cells were fixed 12 hr after isolation and stained for BrdU incorporation. Approximately 6.5% of the cells were positive for BrdU, indicating that *in vivo* dividing SN progenitor cells represented a significant proportion of the isolated cells. The percentage of NG2/

BrdU double-labeled cells was similar to that in the *in vivo* data (50%). No neuronal markers (β-tubulin III) and only occasional mature glial markers (S100β) were observed among the isolated cells (data not shown).

Lineage potential of SN progenitor cells

Richards and Colleagues determined the neural lineage potential of SN progenitor cells. Isolated progenitor cells were cultured in the presence of either FGF2, which has been described as a necessary mitogen for maintaining multipotent progenitor cells *in vitro* or FGF8, which is a mitogen for progenitor cells in the developing midbrain. To determine whether these two growth factors were able to stimulate the proliferation of progenitor cells, BrdU was added at 5 d *in vitro* (DIV) to determine the percentage of dividing cells. At 7 DIV, > 90% of the FGF-treated cells had incorporated BrdU, whereas < 10% of the cells in control cultures that were grown without the addition of growth factors were BrdU-positive. In addition, control cultures displayed signs of increased cell death, such as fragmented cell nuclei (data not shown). Growth rates of FGF2- and FGF8-treated cultures were comparable (data not shown). This demonstrates that FGF2, as well as FGF8, can stimulate the proliferation of SN progenitor cells. The neural lineage potential of SN-derived progenitors was evaluated by culturing isolated cells in medium containing FGF2 or FGF8 for 7 d. Cells were then fixed and stained for expression of lineage-associated markers. At this time point, cells in both cultures abundantly expressed markers for immature precursors such as nestin, a marker for immature neuroepithelial precursors, and the glial precursor marker Moreover, low percentages of GFAP-positive astrocytes and β-tubulin III-positive neurons were observed in both culture conditions. No RIP-positive oligodendrocytes were detected in the cultures. After retinoic acid-induced differentiation for 7 days FGF2- and FGF8-treated cultures contained a significantly higher number of cells that had differentiated into neurons (17% in FGF2-stimulated cultures 18% in FGF8-stimulated cultures), astrocytes (6% and 17%, respectively) and oligodendrocytes (2% in both conditions) Cultures were also treated with BrdU for 48 hr before differentiation. After differentiation, the majority (>85%) of the neurons and glia were labeled for BrdU, demonstrating that these cells were generated de novo from a dividing cell population. Together, these results demonstrate that SN-derived progenitor cells are able to generate cells from all three neural. Cultured SN progenitor cells give rise to all three neural lineages *in vitro*. β-tubulin III positive-neurons (A; green), GFAP-positive astrocytes (A; red), and RIP-positive oligodendrocytes (B; red) are observed after differentiation. A, B, Nuclei Next, we determined whether SN-derived progenitor cells are multipotent or restricted to a single neural lineage using a previously described clonal analysis approach (Palmer., *et al*. 1999). In brief, proliferating cells were infected with low-titer GFP retroviruses; single GFP-positive cells and their progeny were closely monitored during an initial 7 d proliferation phase and subsequent 7 d differentiation phase. Clones were then evaluated for differentiation into neurons and glia. Some clones generated only cells of a single neural lineage. However, a significant proportion of clones in both culture conditions contained both neurons and glia demonstrating that multipotent progenitor cells are present in these cultures. In addition, these results confirm that neurons and glia are de novo generated from proliferating cells. Phenotypic characterization of differentiated clonal SN progenitor cells propagated in the presence of FGF2.

Progenitor cells in the SN differentiate into glia 4 weeks after the final BrdU injection. A, Colocalization of a BrdU-positive nucleus (blue) with the oligodendrocyte marker APC (green). B, Colocalization of a BrdU-positive nucleus (blue) with the astrocyte marker S100_ (green). C, Many BrdU-positive nuclei (blue) appear to be associated with dopaminergic neurons (red). Three-dimensional reconstruction in different planes (insets) revealed that these nuclei belong to satellite cells that are in close proximity to the dopaminergic neurons. S100_ is shown in green. A-C, TH is shown in red below.

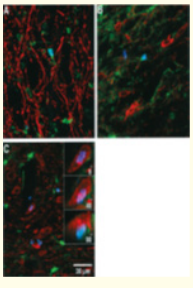

Figure 3

Lineage potential of individual SN progenitor cells. Cells were infected with low-titer GFP retrovirus. Individual infected cells and their progeny were differentiated after a 7d proliferation period in FGF8-supplemented media (A) or FGF2-supplemented. We subsequently determined whether the neuronal potential of progenitor cells is confined to quiescent progenitor cells from the SN or is also a property of actively dividing cells in this region. Animals were injected with BrdU (200 mg/kg) on 7 consecutive days before isolation. Cells were isolated and immediately differentiated for 7d

using different paradigms. In all conditions, 1 - 2% of the BrdU-positive cells were labeled for the neuronal marker β-tubulin III, demonstrating that at least a proportion of actively *in vivo* dividing cells or their progeny have an intrinsic potential to differentiate into neurons when removed from their *in vivo* environment. *In vivo* proliferating SN progenitor cells have an intrinsic neuronal potential. Proliferating progenitor cells were labeled *in vivo* by injection of BrdU. SN progenitor cells were differentiated immediately after isolation. β-tubulin III-positive *In vivo* neuronal differentiation potential of SN progenitor cells because the possibility remains that SN progenitor cells can only differentiate into neurons under *in vitro* conditions and that their potential *in vivo* is much more restricted, we subsequently determined whether SN progenitor cells can differentiate into neurons *in vivo*. The adult hippocampus has been demonstrated to provide signals that can direct multipotent cells from different CNS regions toward a neuronal fate (Suhonen and Shihabuddi, 2000). We took advantage of this property of the hippocampus to determine whether SN-derived progenitor cells have the potential to differentiate into neurons *in vivo*. Progenitor cells were freshly isolated and cultured in FGF2 or FGF8 for 6 d. This brief *in vitro* period removed dying cells from the progenitor cell preparation and allowed the expansion of SN progenitor cells to a sufficient number for the transplantation experiment. Proliferating progenitor cells were labeled with BrdU 2d before transplantation. Cells were stereotactically injected into the hilar region of the hippocampus. Animals were analyzed 3 weeks later. The distribution of the grafted cells was similar for FGF2- and FGF8-treated cells. The majority of the grafted cells was found close to the grafting site in the hilus or the neuronal layers of the dentate gyrus. However, BrdU-positive cells were observed up to 1.5 mm along the AP axis and 2.5 - 3 mm along the ML axis, indicating limited migration of grafted cells. Approximately 20% of grafted cells (160 cells of a total of 800 grafted cells examined) in the neuronal layers of the dentate gyrus expressed early and/or mature neuronal markers such as β-tubulin III and Neu.

Discussion

Many studies has suggested that neural progenitor cells persist throughout adulthood in diverse areas of the CNS, there has been little evidence for the existence of progenitor cells in the adult SN. Potter and colleagues hypothesized fetal neural progenitor cells have been isolated repeatedly from the mesencephalon and have been used in experimental cell-replacement approaches for PD and later experiment revealed the presence of actively dividing cells in the SN that are able to give rise to oligodendrocytes and astrocytes *in vivo*. These newly generated glial cells were not observed immediately after the BrdU pulse but only 4 weeks after the final BrdU injection. This delay suggests that these cells are derived from more immature progenitor cells that have differentiated into mature glial cells and not from dividing mature glia. Almost 50% of the BrdU-labeled cells were associated with the glial progenitor marker immediately after the BrdU pulse and the percentage of NG2/BrdU-labeled cells tended to decrease over time. It is therefore possible that the newly generated glial cells in the SN are the progeny of NG2-positive progenitors. Gensert and colleagues therefore stated that the adult SN displays characteristics that are different from the adult cortex, where NG2 cells constitute only a minor proportion of dividing cells, but resembles the adult spinal cord where NG2-positive progenitor cells are the major dividing cell population and mature glial cells are generated from immature dividing cells. The phenotype of the remaining proliferating cells remains unclear. Horner experimental studies shows endothelial cells proliferate within the CNS and our preliminary results indicate that indeed some of the BrdU-labeled cells colocalize with endothelial cell markers. However, most of the BrdU-positive cells of unknown phenotype were not found in the walls of blood vessels, which suggests a phenotype other than endothelial cells. The presence of newborn glial cells in the adult SN suggests that constant glial cell replacement is taking place in this region. Previous findings that glial cells are key regulators of synaptic transmission and extracellular homeostasis in the CNS underline the importance of the maintenance of the glial population [3]. Future experiments need to address the functional relevance of gliogenesis in the SN for the maintenance of the glial population. Despite careful cell-by-cell analysis, we have no indication that neurogenesis occurs either in the intact or in the lesioned SN. Multiple BrdU-positive nuclei were closely associated with dopaminergic neurons. However, detailed analysis of these nuclei (a total of 800 cells) by three-dimensional confocal analysis clearly demonstrated that these nuclei without exception belonged to cells that were closely attached to the dopaminergic neurons. The proximity of newborn cells with mature existing neurons has been observed in different areas of the adult CNS [4,5]. The significance of this phenomenon is not known; however, one could speculate that this close association is necessary for cross talk between those cells, for induction of differentiation, or for trophic support. Our failure to detect neurogenesis in the SN is consistent with a previous study in which cell proliferation but no generation of new neurons after1-methyl-4-phenyl-1,2,3,6tetrahydropyridine lesion was observed [6]. We cannot rule out the possibility that neurogenesis in the SN is an extremely low-frequency event that escapes detection by our BrdU-injection paradigm. However, in our paradigm we used BrdU at a concentration in the range of a dose that allowed the detection of a large pool of

proliferating cells and their progeny in the adult hippocampus [7], making it unlikely that any newly generated neurons were not labeled with BrdU. A previously described progenitor cell enrichment protocol (Palmer., *et al*. 1999) enabled us to further characterize SN progenitor cells. In striking contrast to their glial-restricted differentiation pattern *in vivo*, isolated SN progenitor cells differentiated into all neural lineages including neurons *in vitro*. Interestingly, the ability of SN progenitor cells to differentiate into neurons was observed in progenitor cells that had proliferated previously *in vivo*. Another important point is that *in vivo* proliferating SN progenitor cells could differentiate into neurons immediately after isolation, without short-term exposure to high doses of FGF2 which in previous *in vitro* studies led to neuronal differentiation of glial-restricted precursors (Palmer and Raff, 2000). Together, these results suggest that at least a subpopulation of proliferating progenitor cells in the adult SN have an intrinsic neurogenic potential that is suppressed by the local environment. At this point it is not clear which population of *in vivo* dividing cells has this potential. Previous studies have proposed that new neurons are derived from multipotent GFAP-positive cells [8] or ependymal cells [9] within neurogenic areas of the adult CNS. However, our dissection approach (tissue adjacent to the ventricle was completely removed) rules out the possibility that ependymal cells were the *in vivo* proliferating cells that gave rise to neurons in culture immediately after isolation, and we have not found evidence for proliferating GFAP-positive cells in the adult SN. A small subset of *in vivo* dividing cells expressed nestin, a marker for multipotent neural progenitor cells during development [10]. It is possible that the proliferating nestin-positive cells in the adult SN correspond to the multipotent cells detected in our clonal analysis, and that these cells are giving rise to neurons after isolation. The future identification of definite markers for adult multipotent CNS progenitor cells will be very helpful in clarifying this point. We were also able to demonstrate that the neuronal differentiation potential of SN progenitor cells is not confined to*in vitro* conditions but extends to the *in vivo* situation. SN progenitor cells that were transplanted into the adult hippocampus were able to survive at least 3 weeks in this location and expressed markers of both immature and mature neurons. Because grafted cells were labeled with BrdU just before transplantation, these neurons were exclusively derived from proliferating progenitor cells and not from cells that had already differentiated into postmitotic neurons *in vitro*. Some of the SN progenitor cell-derived neurons were found in deeper layers of the dentate granular cell layer, indicating the possibility that they had integrated into the hippocampal circuit. To investigate this possibility, our future experiments will address whether these neurons receive synaptic inputs and make functional connections. In previous experiments, we have shown that multipotent progenitor cells from different CNS regions generate neurons after transplantation into the adult hippocampus (Suhonen and Shihabuddin, 2000). These cells were cultured for > 2 years before transplantation, increasing the likelihood for transformation and/or alterations of their native characteristics (Palmer., *et al*. 1997). It is important to note that in the present experiments, the transplanted progenitor cells were only cultured for 6 d and yet were able to differentiate into neurons. This finding indicates that prolonged culture is not a prerequisite for adult progenitor cells from non-neurogenic regions to differentiate into neurons in a heterotopic neurogenic environment. Moreover, it adds to the existing evidence that the adult hippocampus provides general proneuronal signals that can be interpreted by adult progenitor cells from CNS regions as different as the hippocampus, spinal cord, and SN. One striking observation is that, in contrast to their hippocampus-grafted counterparts, progenitor cells derived from either the adult SN (this study) or the adult hippocampus (data not shown) did not differentiate into neurons after transplantation into the SN. These findings strongly emphasize the importance of the environment for neurogenesis and suggest that proneuronal signals are absent and/or that inhibitory signals of neuronal differentiation are present in the adult SN. The presence or absence of these signals also would explain our observation that although progenitor cells differentiate only into glial phenotypes *in situ*, they are able to differentiate immediately into neurons after removal from the SN and exposure to proneuronal signals [11]. Exposure to FGF2 or FGF8 was not required for neuronal differentiation of *in vivo* proliferating progenitor cells but was necessary for efficient recruitment of SN progenitor cells into the cell cycle and maintenance of these cells in culture. Our preliminary results suggest that neither of the factors is expressed at high levels in the adult SN. It is possible that delivery of FGF2 or FGF8 into the adult SN increases the proliferation of endogenous multipotent progenitor cells, thereby increasing the pool of cells with a neuronal potential. However, the failure of FGF2- or FGF8-treated cells to differentiate into neurons after transplantation into the SN indicates that these two factors are not sufficient to promote *in situ* neuronal differentiation of SN progenitors. In a recent report [12], demonstrated that bone morphogenetic protein-4 (BMP4) inhibits neuronal differentiation of adult multipotent progenitor cells *in vivo* and promotes glial differentiation. The BMP-antagonist Noggin is highly expressed close to the neurogenic subventricular zone and promotes neuronal differentiation by blocking the gliogenic actions of BMP4. It is possible that this antagonism between BMP4 and Noggin regarding the neural fate of progenitor cells holds true for

other CNS regions, including the adult SN, and that lack of Noggin expression is in part responsible for the exclusive glial differentiation of SN progenitor cells *in situ* [13-33].

Conclusion

The adult SN contains neural progenitor cells with the potential to differentiate into neurons. Some cells with a neuronal differentiation potential are readily proliferating under physiological conditions, which opens up the possibility that provision of proneuronal differentiation signals might be sufficient to drive these cells down a neuronal lineage.

Further Research

Future experiments need to be directed at the characterization and development of the microenvironment of the SN as a first step to generating a permissive environment for neuronal differentiation. In addition, signals need to be identified that can direct the differentiation of adult SN progenitor cells toward a dopaminergic neurotransmitter.

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