



Grafted Subventricular Zone Neural Stem Cells Display Robust Engraftment and Similar Differentiation Properties and Form New Neurogenic Niches in the Young and Aged Hippocampus

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Key Words. Cell transplantation • Cellular therapy • Hippocampal neurogenesis • Neural stem cells • Stem cell grafts • Subventricular zone stem cells

ABSTRACT

As clinical application of neural stem cell (NSC) grafting into the brain would also encompass aged people, critical evaluation of engraftment of NSC graft-derived cells in the aged hippocampus has significance. We examined the engraftment and differentiation of alkaline phosphatase-positive NSCs expanded from the postnatal subventricular zone (SVZ), 3 months after grafting into the intact young or aged rat hippocampus. Graft-derived cells engrafted robustly into both young and aged hippocampi. Although most graft-derived cells pervasively migrated into different hippocampal layers, the graft cores endured and contained graft-derived neurons expressing neuron-specific nuclear antigen (NeuN) and γ -amino butyric acid in both groups. A fraction of migrated graft-derived cells in the neurogenic subgranular zone-granule cell layer also expressed NeuN. Neuronal differentiation was, however, occasionally seen amid graft-derived cells that had migrated into non-neurogenic regions, where substantial fractions differentiated into S-100 β + astrocytes, NG2+ oligodendrocyte progenitors, or Olig2+ putative oligodendrocytes. In both age groups, graft cores located in non-neurogenic regions displayed many doublecortin-positive (DCX+) immature neurons at 3 months after grafting. Analyses of cells within graft cores using birth dating and putative NSC markers revealed that DCX+ neurons were newly born neurons derived from engrafted cells and that putative NSCs persisted within the graft cores. Thus, both young and aged hippocampi support robust engraftment and similar differentiation of SVZ-NSC graft-derived cells. Furthermore, some grafted NSCs retain the “stemness” feature and produce new neurons even at 3 months after grafting, implying that grafting of SVZ-NSCs into the young or aged hippocampus leads to establishment of new neurogenic niches in non-neurogenic regions. *STEM CELLS TRANSLATIONAL MEDICINE* 2016;5:1–12

SIGNIFICANCE

The results demonstrate that advanced age of the host at the time of grafting has no major adverse effects on engraftment, migration, and differentiation of grafted subventricular zone-neural stem cells (SVZ-NSCs) in the intact hippocampus, as both young and aged hippocampi promoted excellent engraftment, migration, and differentiation of SVZ-NSC graft-derived cells in the present study. Furthermore, SVZ-NSC grafts showed ability for establishing neurogenic niches in non-neurogenic regions, generating new neurons for extended periods after grafting. This phenomenon will be beneficial if these niches can continuously generate new neurons and glia in the grafted hippocampus, as newly generated neurons and glia are expected to improve, not only the microenvironment, but also the plasticity and function of the aged hippocampus. Overall, these results have significance because the potential application of NSC grafting for treatment of neurodegenerative disorders at early stages of disease progression and age-related impairments would mostly involve aged persons as recipients.

INTRODUCTION

Neural stem/progenitor cells (NSCs) competent for creating new neurons and glia endure in the adult and aged brain. Such NSCs are particularly conspicuous in neurogenic regions of the brain,

such as the anterior subventricular zone (SVZ) of the forebrain [1, 2] and the subgranular zone (SGZ) of the dentate gyrus (DG) in the hippocampus [3–7]. Enhanced NSC activity in response to injury is a conspicuous feature in the young and adult brain, which results in increased production

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of new neurons. Such increased neurogenesis in the SVZ after conditions such as stroke or traumatic brain injury has been shown to be reparative in many studies [8–13]. However, the functional consequences of increased NSC activity and neurogenesis in the DG after brain injury are largely unknown.

Studies in certain disease models have suggested that injury-induced increased DG neurogenesis is useful for partial self-repair and/or maintenance of cognitive function. In contrast, studies in other models such as seizures have suggested detrimental consequences for hippocampus function because of the abnormal integration of newly born neurons [14–19]. Furthermore, the response of NSCs to injury in the aged brain, in particular, in the hippocampus, has been found to be greatly impaired [20, 21]. Additionally, no evidence is available to date for wide-ranging functional recovery with spontaneous replacement of degenerated neurons by new neurons produced from endogenous NSCs in the DG, as NSC-derived neurons seem to differentiate only into DG granule cells even when substantial neurodegeneration is present in the adjoining CA3 region [22, 23]. Thus, aging, Alzheimer's disease, or injury-related neurodegeneration in the hippocampus is not followed by adequate self-repair [20, 21, 24–28]. Therefore, testing the survival, differentiation, and beneficial effects of grafting of NSCs expanded from diverse sources in the developing and adult brain into the adult and aged hippocampus under intact and injured conditions has considerable significance.

Multipotent NSCs from the developing and adult brain that could be expanded in culture as neurospheres using mitogens such as fibroblast growth factor-2 (FGF-2) and epidermal growth factor (EGF) are one of the donor cell types considered suitable for grafting in neurodegenerative disorders [29, 30]. This is because such NSCs can be expanded in culture for extended periods from diverse sources [1, 31–33]. Moreover, newly developed technologies allow direct conversion of somatic cells, such as fibroblasts, lymphocytes, or glia, into multipotent NSCs [34–36], which could facilitate grafting of patient-specific NSCs in the future. Thus, interest is great in ascertaining the engraftment, differentiation, and integration of NSCs in the adult brain using distinct animal models of neurological disorders. Yet, as clinical application of neural cell grafting would also include elderly people afflicted with neurodegeneration, analyses of the potential of different NSCs for engraftment and differentiation in the aged brain are necessary. Therefore, in the present study, we quantified the engraftment, distribution, and differentiation of EGF- and FGF-2-responsive NSCs isolated from the anterior SVZ after grafting into the intact hippocampus of young (3–4-month-old) and aged (22–24-month-old) rats. The donor NSCs were isolated from postnatal day 2 (PND2) rat pups expressing alkaline phosphatase (AP) transgene in all cells, expanded in vitro as neurospheres, and labeled with 5'-bromodeoxyuridine (BrdU). Graft-derived cells were analyzed 3 months after grafting using markers of neurons, astrocytes, oligodendrocyte progenitors, oligodendrocytes, and putative NSCs. In addition, the potential proliferation of graft-derived cells was assessed 1 month after grafting through dual and triple immunofluorescence methods using graft cell, birth dating, and/or putative NSC markers.

MATERIALS AND METHODS

Animals

Young adult (3–4-month-old) and aged (22–24-month-old) Fischer 344 rats were obtained from Harlan Sprague Dawley (Indianapolis,

IN, <http://www.harlan.com>). The institutional animal care and use committees of the Duke University Medical Center, Durham Veterans Affairs Medical Center (Durham, NC), Texas A&M Health Science Center College of Medicine, and Central Texas Veterans Health Care System (Temple, TX) approved all the procedures performed in the present study. Supplemental online Figure 1 illustrates the experimental design used in the present study.

Preparation of Subventricular Zone-Neural Stem Cells for Grafting

The methods used to prepare the SVZ-NSCs are described in the supplemental online data. For grafting, cell suspensions having greater than 80% cell viability were chosen, and live cells were adjusted to a density of 1.00×10^5 viable cells per microliter. The final cell suspension was treated with 20 ng/ml brain-derived neurotrophic factor (BDNF) before grafting; the addition of BDNF to the final cell suspension enhances the survival and differentiation of graft-derived cells in the host hippocampus [37]. The cells were maintained at 4°C throughout the grafting procedure.

In Vitro Differentiation Studies of Subventricular Zone-Neural Stem Cells

In order to examine the differentiation potential of neurosphere cells expanded from SVZ-NSCs that were used for grafting, neurosphere cells were incubated in culture dishes coated with poly-D-lysine and containing Dulbecco's modified Eagle's medium, F12, and B-27 nutrient mixture for 4–7 days, as described previously [29, 30]. The cultures were stained for identification of β -III tubulin (TuJ-1)-positive neurons, γ -amino butyric acid-positive (GABA+) inhibitory neurons, glial fibrillary acidic protein-positive (GFAP+) astrocytes, and RIP+ oligodendrocytes, using single and dual immunofluorescence methods described in our previous reports [30, 38]. The antibodies used are listed in supplemental online Table 1.

Grafting of Subventricular Zone-Neural Stem Cells Into the Young and Aged Hippocampus

The methods used for grafting are detailed in the supplemental online data. One microliter of SVZ-NSC suspension comprising ~100,000 live cells was injected into each of the three sites in the hippocampus (i.e., 300,000 live cells in three transplants for each hippocampus) in several spurts over a period of 1–2 minutes [20, 37, 39, 40]. Based on the BrdU-labeling index (92.1% of all cells) in the cell suspension, this amounted to grafting of 92,100 BrdU+ cells per graft and 276,300 BrdU+ cells per hippocampus.

Tissue Processing

Three months after grafting, rats belonging to both young adult and aged groups underwent intracardiac perfusions with 4% paraformaldehyde. The brains were dissected, postfixed in 4% paraformaldehyde overnight, and treated with increasing concentrations (10%, 20%, and 30%) of sucrose solution. Thirty-micrometer-thick sections were cut coronally through the entire hippocampus using a cryostat and collected serially in 24-well plates containing phosphate buffer.

Quantification of Engrafted Graft-Derived Cells

Serial sections (every 10th) from the grafted animals ($n = 5$ per group) were first processed for AP or BrdU immunostaining as described in our earlier reports [37, 40–42]. The antibodies used are

listed in supplemental online Table 1. We used AP immunostaining to identify the graft cores and migrated graft-derived cell clusters in the hippocampus. Because AP is expressed diffusely in membranes and cytoplasm, individual graft-derived cells could not be ascertained using light microscopy, however. Therefore, we chose BrdU-immunostained sections for quantification of the approximate numbers of engrafted cells, as BrdU immunostaining clearly demonstrated nuclei of graft-derived cells that retained BrdU. Cells positive for BrdU were counted in serial sections through the entire anteroposterior extent of the hippocampus using the optical fractionator counting method in a StereoInvestigator system (MBF Bioscience, Williston, VT, <http://www.mbfbioscience.com>) comprising a color digital video camera (Optronics Inc., Muskogee, OK, <http://www.optronicsinc.com>) interfaced with a Nikon E600 microscope (Nikon, Tokyo, Japan, <http://www.nikon.com>), by employing methods described in our earlier reports [37, 43]. Additional details on counting methods are available in the supplemental online data. The overall engraftment in each age group is expressed as the number of BrdU+ graft-derived cells recovered per hippocampus.

Analyses of the Presence of Microglia/Macrophages Among BrdU+ Structures

To determine whether a significant fraction of BrdU immunoreactive structures or elements represented microglia or macrophages that had ingested BrdU material from dead cells, we quantified the percentages of BrdU+ elements/structures found inside ionized calcium binding adaptor molecule 1-positive (IBA-1+) microglia using BrdU and IBA-1 dual immunofluorescence and Z-section analyses in a confocal microscope. The antibodies used are listed in supplemental online Table 1.

Analyses of Graft Cell Differentiation in the Host Brain

We quantified the phenotype of graft-derived cells through dual immunofluorescence and confocal microscopy for BrdU or AP with distinct neural cell antigens. The neural cell antigens included markers of (a) neurons (Tuj-1); (b) mature neurons (neuron-specific nuclear antigen [NeuN]); (c) inhibitory interneurons (GABA); (d) astrocytes (GFAP); (e) mature astrocytes (S-100 β); (f) oligodendrocyte progenitors (NG2); and (g) putative oligodendrocytes (Olig2). The dual immunofluorescence methods used have been described in our earlier reports [4, 37, 42, 44]. The antibodies used are listed in supplemental online Table 1. Dual-labeled cells were quantified through Z-section analyses using an Fv10i confocal microscope (Olympus, Tokyo, Japan, <http://www.olympus-ims.com>). For assessment of each neural cell antigen expression, four to six sections through the hippocampus were examined in every animal belonging to the two age groups ($n = 4$ per group).

Analyses of Newly Born Neurons Within Graft Cores

The possible production of new neurons within the graft cores located in non-neurogenic regions of the hippocampus, at an extended time point after grafting, was examined through transplantation of SVZ-NSCs derived from transgenic mice expressing alkaline phosphatase transgene in all cells (AP transgenic mice) and subsequent dual immunofluorescence analysis of hippocampal tissues for AP and DCX. The antibodies used are listed in supplemental online Table 1. Young and aged animals perfused 3 months after grafting were used for this analysis.

Analyses of Proliferating Cells and Putative NSCs Among Graft-Derived Cells

Both proliferation of graft-derived cells and neuronal differentiation of new cells produced by grafts were assessed in additional subgroups of animals ($n = 4$ per group) through previous labeling of donor NSCs (i.e., at the time of grafting) with chlorodeoxyuridine (CldU) and subsequent labeling of proliferating grafted cells via injections of iododeoxyuridine (IdU; 12 daily injections at 50 mg/kg), 1 month after grafting. The animals were perfused 24 hours after the last IdU injection, and the brain tissues were processed for IdU and DCX dual immunofluorescence and CldU, IdU, and DCX triple immunofluorescence. Using Z-section analyses in a confocal microscope, the generation of new cells and neurons by graft-derived cells within graft cores (i.e., IdU+ cells expressing DCX and CldU+ and IdU+ cells expressing DCX) were confirmed. In addition, the presence of putative NSCs within the graft cores was examined using triple immunofluorescence for AP, GFAP, and Sox-2. The antibodies used are listed in supplemental online Table 1.

Statistical Analysis

All data are expressed as the mean \pm SEM. The data from young adult and aged rats ($n = 4$ –5 animals per group) were compared using a two-tailed, unpaired Student *t* test.

RESULTS

In Vitro Differentiation of SVZ-NSCs

Neurosphere cells expanded from anterior SVZ-NSCs differentiated into Tuj-1+ neurons, GFAP+ astrocytes, and RIP+ oligodendrocytes (supplemental online Fig. 2A1–B3). The percentages of primary neurosphere cells that differentiated into Tuj-1+ neurons (~24%), GFAP+ astrocytes (~70%), S-100 β + mature astrocytes (53%), and O4/RIP+ oligodendrocytes (~12%–14%) after 7 days of incubation in differentiation medium have been described in our previous report [30]. A vast majority of Tuj1+ neurons (~90%) derived from SVZ-NSCs also expressed the inhibitory neurotransmitter GABA (supplemental online Fig. 2C1–C3). Thus, SVZ-NSCs expanded from PND2 brains showed a propensity for differentiating mostly into astrocytes and GABA-ergic neurons in standard culture conditions.

Distribution and Engraftment of Graft-Derived Cells

Labeling of SVZ-NSCs with BrdU before grafting facilitated examination of the distribution or migration of individual graft-derived cells in the host hippocampus, and the presence of AP transgene allowed identification of graft cores and clusters of migrating cells. Examination of SVZ-NSC graft-derived cells using BrdU immunostaining at 3 months after grafting revealed the engraftment and widespread migration of graft-derived cells in both young and aged animals (Fig. 1A1–1B4). However, smaller graft cores persisted in areas such as the hippocampal fissure, the CA1 stratum radiatum or the junction of DG, the CA3b subregion, and the lateral ventricle in both age groups of animals (Fig. 1A1, 1A2, 1B1, 1B2). Supplemental online Figures 3 and 4 illustrate the locations of graft cores in additional young and aged animals. AP immunostaining also revealed graft cores in these regions (data not illustrated). Graft-derived cells in both young and aged hosts migrated into neurogenic and non-neurogenic regions of the

hippocampus. A significant number of engrafted cells could be seen in the subgranular zone and/or granule cell layer of the DG (Fig. 1A4, 1B4). Such migrated cells could also be observed with AP-NeuN dual immunofluorescence (supplemental online Fig. 5).

Quantification of the number of BrdU+ graft-derived cells through BrdU immunostaining of serial sections through the entire hippocampus and stereological counting of BrdU+ cells revealed that the overall engraftment of cells derived from SVZ-NSC grafts into the hippocampus was robust in both age groups (equivalent to 240%–262% of injected cells; Fig. 1C1). To verify that greater fractions of BrdU+ structures did not represent microglia or macrophages that have ingested BrdU material from dead cells, we quantified the percentages of BrdU+ elements/structures found inside IBA-1+ microglia (Fig. 1D1–1E3). This analysis revealed that <5% of BrdU+ structures were found inside the IBA-1+ microglia in both young and aged hippocampi (Fig. 1F), implying that a vast majority of BrdU+ structures represent graft-derived cells that have differentiated into neurons or glia in both age groups. However, measurement of the BrdU+ graft cell number does not provide an accurate quantification of the absolute graft cell yield, as dilution of BrdU in some grafted NSCs is conceivable owing to their division more than a few times after grafting. Because the progeny of graft-derived NSCs lacking BrdU were not accounted for in our BrdU+ graft cell counts, the graft cell yield derived was likely underestimated in both groups. It is also possible that one environment (e.g., young hippocampus) promotes increased proliferation of NSCs than the other environment (e.g., aged hippocampus), leading to loss of the BrdU label in greater fractions of graft-derived cells. Hence, the BrdU+ graft cell counts cannot be considered as absolute graft cell yields. Nonetheless, considering that the recovery of BrdU+ cells in both young and aged hippocampi (662,772–722,554 cells per hippocampus; Fig. 1C1) is considerably higher than the numbers of BrdU+ NSCs initially grafted (276,300 live BrdU+ cells through three grafts per hippocampus), the results do suggest that the environment in both young and aged hippocampi is conducive for engraftment of greater fractions of NSC graft-derived cells. Furthermore, the volume of hippocampus and the adjoining brain regions containing graft-derived cells was found to be similar between the young and aged animals (Fig. 1C2), suggesting that advanced host age at the time of grafting did not negatively affect the migration of cells derived from SVZ-NSCs grafts in the hippocampus.

Differentiation of BrdU+ Graft-Derived Cells Into Neurons and Glia

We examined the neuronal differentiation of BrdU+ graft-derived cells that remained in the graft core, that migrated into the subgranular zone-granule cell layer (SGZ-GCL) of the DG, and that invaded into all other regions of the hippocampus (non-neurogenic regions), using BrdU-NeuN dual immunofluorescence and confocal microscopy (Fig. 2). Differentiation of some graft-derived cells into NeuN+ mature neurons could be clearly seen in graft cores located in both young and aged hippocampi (Fig. 2A1–2B3). Quantification revealed that the overall differentiation of graft-derived cells into NeuN+ mature neurons was 15%–17% for graft cores, which did not vary between the young and aged hosts (Fig. 2F). A fraction of graft-derived cells that migrated into the SGZ-GCL also

differentiated into NeuN+ mature neurons in both young and aged hippocampi (Fig. 2C1–2D3). Measurement of BrdU-NeuN dual-labeled cells among all BrdU+ cells revealed that 31% of graft-derived cells differentiated into NeuN+ mature neurons in the SGZ-GCL of the young hippocampus (Fig. 2F). However, in the SGZ-GCL of the aged hippocampus, only 20% of graft-derived cells differentiated into NeuN+ neurons (Fig. 2F), implying that the aged SGZ-GCL is less conducive for neuronal differentiation of NSC graft-derived cells. Neuronal differentiation was also observed occasionally among graft-derived cells that migrated into non-neurogenic regions of the hippocampus or into the adjoining cortex (Fig. 2E1–2E3).

Dual immunofluorescence analyses for BrdU and GABA revealed differentiation of graft-derived cells into GABA-ergic neurons in both young and aged hippocampi (Fig. 3A1, 3A2). Quantification demonstrated that 20%–21% of NSC graft-derived cells differentiated into GABA+ neurons in the young and aged hippocampi, with most such neurons observed in the graft core and the dentate gyrus (Fig. 3A3). Analyses of glia revealed that 36%–41% of graft-derived cells differentiated into S-100 β -positive mature astrocytes (Fig. 4A1–4A3), 48%–51% differentiated into GFAP+ astrocytes (Fig. 4B1–4B3), and 22%–25% differentiated into Olig2+ oligodendrocyte-like cells (Fig. 4C1–4C3). Thus, differentiation of NSC graft-derived cells into neurons and glia did not vary considerably between the hippocampus of young and aged hosts. The only exception is the SGZ-GCL, in which the neuronal differentiation of graft-derived cells somewhat declined in the aged host.

Differentiation of AP+ Graft-Derived Cells Into Neurons and Glia

To further authenticate the graft cell differentiation results obtained with dual immunofluorescence for BrdU and neural antigens, we also examined differentiation of AP+ graft-derived cells within graft cores using dual immunofluorescence methods for AP-NeuN, AP-GABA, AP-S100 β , and AP-NG2 (Fig. 5A1–5A7). Confocal microscopic analyses using Z-sectioning revealed that fractions of AP+ graft-derived cells that differentiate into NeuN+ mature neurons (19%–21%), GABA+ interneurons (21%–23%), S-100 β -positive mature astrocytes (34%–38%), and NG2+ oligodendrocyte precursor cells (22%–24%) were comparable between grafts in the young and aged hippocampi (Fig. 5A7, 5B7, 5C7, 5D7). In addition, the percentages of neurons, GABA-ergic interneurons, and astrocytes among graft-derived BrdU+ or AP+ cells were comparable (Figs. 2–5).

DCX+ Immature Neurons Within Graft Cores Located in Non-Neurogenic Regions

Examination of sections with single DCX immunostaining at 3 months after grafting revealed the presence of large numbers of DCX+ immature neurons within graft cores that were located in both neurogenic and non-neurogenic regions of young and aged hippocampi. The graft cores in the non-neurogenic regions were mostly found in the hippocampal fissure adjoining the CA1 stratum radiatum or in CA1 and CA3 regions (Fig. 6A1–6A3). Clusters of immature DCX+ neurons with conspicuous dendrites of variable length and complexity were observed within graft cores, suggesting that they were likely newly born neurons. We next analyzed sections processed for AP (a transgene expressed in all grafted cells) and DCX dual immunofluorescence, which

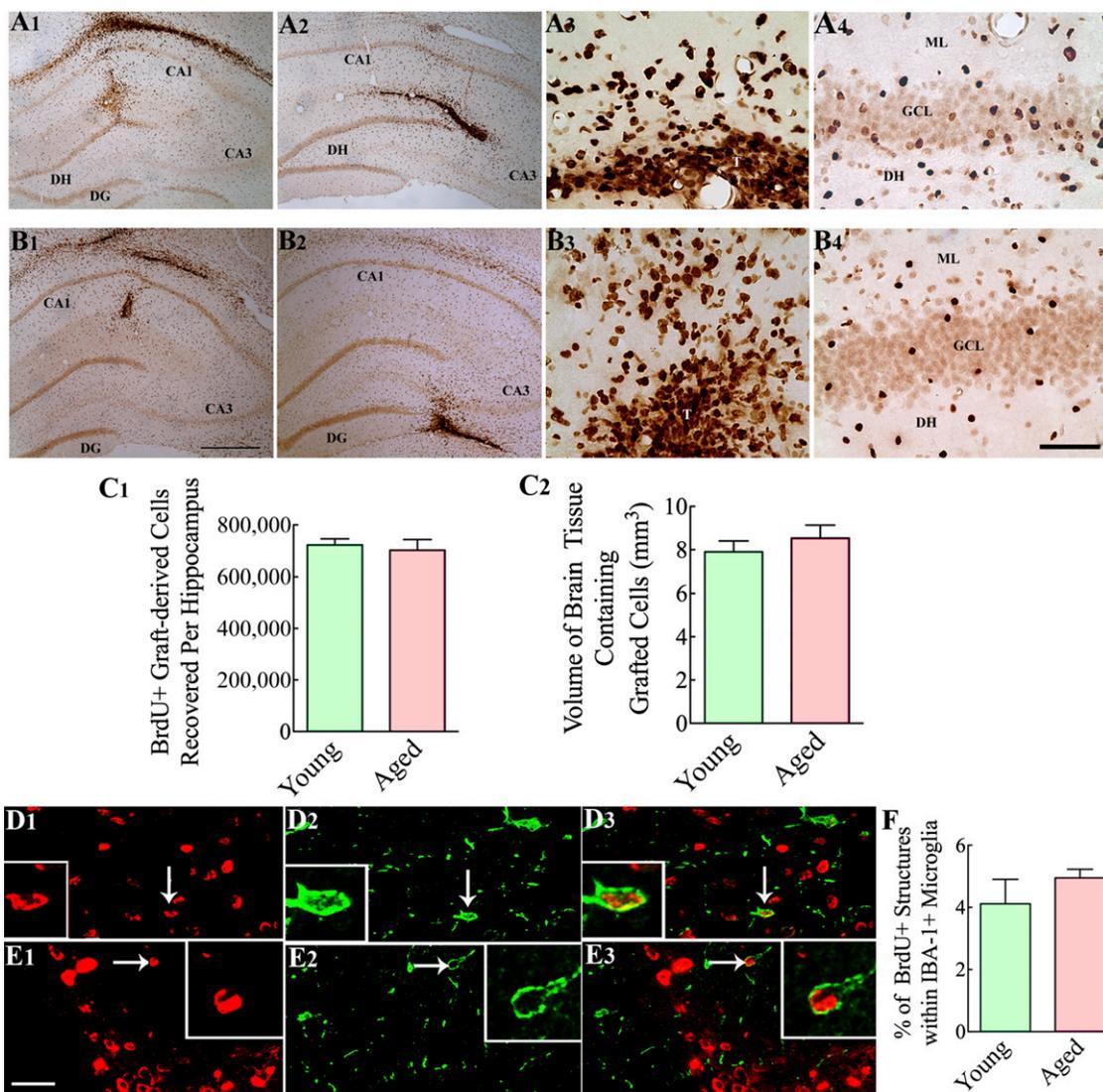


Figure 1. Distribution of BrdU-labeled cells derived from subventricular zone-neural stem cell (SVZ-NSC) grafts in the intact young hippocampi (**A1, A2**) and intact aged hippocampi (**B1, B2**) at 3 months after grafting. (**A3, A4, B3, B4**): Magnified views of regions from (**A2**) and (**B2**), respectively. (**A3, B3**): Graft-derived BrdU+ cells within graft cores and in the surrounding regions. (**A4, B4**): Graft-derived cells that have migrated into the subgranular zone-granule cell layer (SGZ-GCL) of the DG. Scale bars = 500 μ m (**B1**) and 50 μ m (**B4**). Bar charts compare numbers of BrdU+ cells recovered per hippocampus (**C1**) and volumes of brain tissue containing graft-derived cells (**C2**) between the intact young hippocampus and the intact aged hippocampus. (**D1–E3**): The occasional presence of BrdU+ elements inside IBA-1+ cells in the young (**D1–D3**) and aged (**E1–E3**) hippocampi, suggesting that only a very small fraction of BrdU+ elements were debris ingested by microglial cells (arrows). (**D1–E3**, Insets): Magnified views of IBA-1+ microglia containing BrdU+ elements. Scale bar = 25 μ m (**E1**). (**F**): Bar chart depicting fractions of BrdU+ structures found inside IBA-1+ microglia. Abbreviations: BrdU, 5'-bromodeoxyuridine; DG, dentate gyrus; DH, dentate hilus; GCL, granule cell layer; ML, molecular layer.

revealed that virtually all DCX+ immature neurons within the graft cores were positive for AP (Fig. 6B1–6B4), implying that DCX+ immature neurons were generated from grafted cells.

Proliferating Cells and Newly Generated Neurons Within Graft Cores

To examine whether DCX+ neurons found within graft cores were indeed born after grafting, we assessed the proliferation of graft-derived cells through previous labeling of donor NSCs (i.e., at the time of grafting) with CldU and subsequent labeling of proliferating grafted cells via intraperitoneal injections of IdU (12 daily injections at 50 mg/kg), 1 month after grafting into both young and

aged hippocampi. Tissues processed 24 hours after the last IdU injection for IdU and DCX dual immunofluorescence confirmed the generation of newly born neurons within the graft cores (Fig. 6C1–6C6). In both young and aged hippocampi, 39%–40% of DCX+ neurons in the graft core were positive for IdU, and 68%–70% of IdU+ cells expressed DCX (Fig. 6C7, 6C8), implying that the pattern of neurogenesis occurring in the graft cores was comparable between the young and aged hippocampi. Moreover, CldU, IdU, and DCX triple immunofluorescence and confocal microscopic characterization confirmed the proliferation of CldU+ graft-derived cells within graft cores in both young and aged hippocampi (Fig. 7A1–7A4). Some cells positive for both CldU and IdU (i.e., proliferating graft-derived cells) also

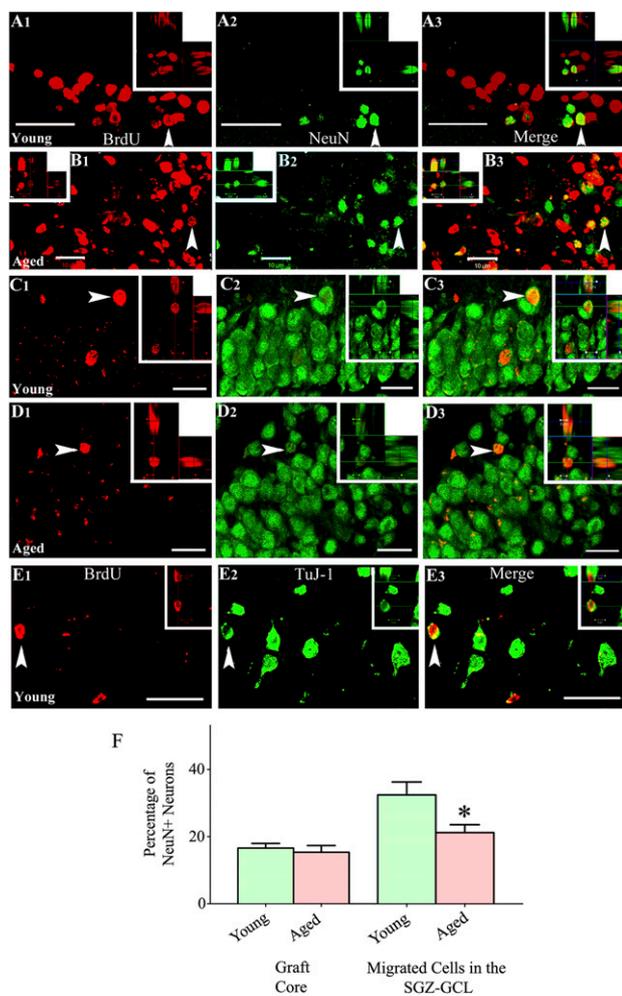


Figure 2. Fractions of cells derived from the SVZ-NSC grafts differentiate into neurons (arrowheads). BrdU+ graft-derived cells (red) differentiate into neurons expressing the NeuN (green) in the intact young hippocampus (A1–A3) and the intact aged hippocampus (B1–B3). (C1–D3): NeuN expression (green) in BrdU+ graft-derived cells (red) that have migrated into the granule cell layer of the intact young hippocampus (C1–C3) and the intact aged hippocampus (D1–D3). (E1–E3): The expression of TuJ-1 (green) among BrdU+ graft-derived cells (red) that have migrated into the non-neurogenic region of the intact young hippocampus. Scale bars = 50 μm (A1–A3, E1, E3) and 25 μm (B1–B3, C1–C3, D1–D3). (F): Bar charts show percentages of graft-derived cells that differentiated into NeuN-positive mature neurons within graft cores and among graft-derived cells that have migrated into the SGZ-GCL of the dentate gyrus in the young and aged hippocampi. The extent of neuronal differentiation is similar in the two age groups for graft cores. However, greater percentages of graft-derived cells differentiated into neurons in the in SGZ-GCL of the young hippocampus ($p < .05$). Abbreviations: BrdU, 5'-bromodeoxyuridine; NeuN, neuron-specific nuclear antigen; SGZ-GCL, subgranular zone-granule cell layer; SVZ-NSC, subventricular zone-neural stem cell; TuJ-1, β -III tubulin.

expressed DCX (Fig. 7A1–7B4), denoting neuronal differentiation of cells born from graft-derived cells. In addition, the graft cores displayed the presence of triple-labeled cells expressing AP (graft cell marker), GFAP (NSC marker), and Sox-2 (another NSC marker), suggesting that putative NSCs reside inside graft cores located in both young and aged hippocampi (Fig. 7C1–7D4).

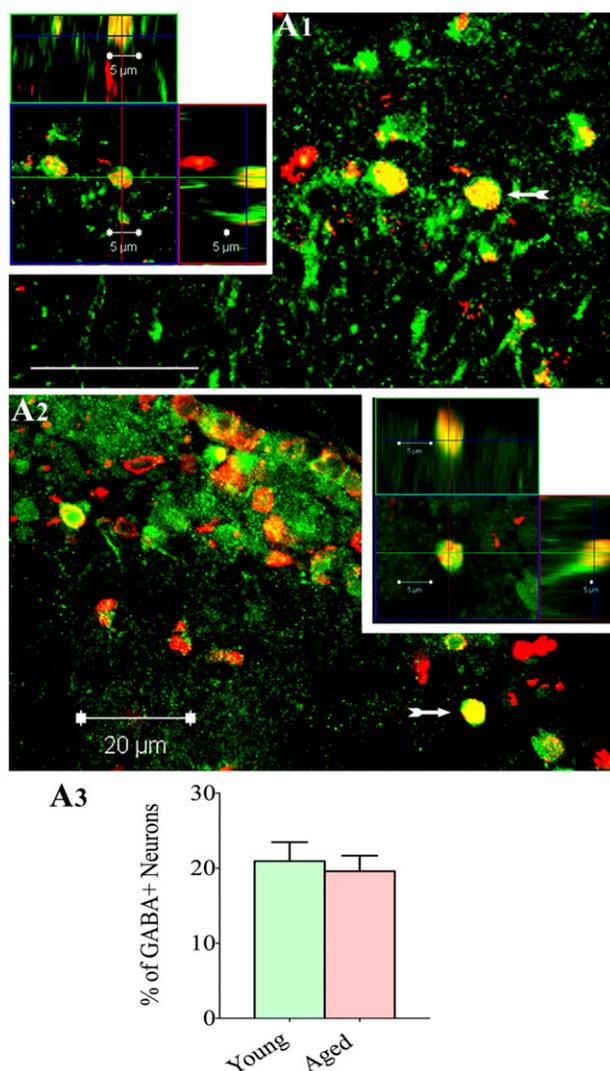


Figure 3. Fractions of cells derived from the subventricular zone-neural stem cell grafts differentiated into GABA+ neurons in the intact young (A1) and aged (A2) hippocampi. (Insets): Orthogonal views of GABA+ neurons indicated by arrows in A1 and A2. Scale bars = 40 μm (A1) and 20 μm (A2). (A3): Bar chart shows percentages of graft-derived cells that differentiated into GABA+ neurons in young and aged hippocampi. Abbreviation: GABA, γ -amino butyric acid.

DISCUSSION

These results provide novel evidence that NSCs from the anterior SVZ display predominantly similar engrafting and differentiation properties when transplanted into the intact young or aged hippocampus. Multiple parameters of graft cell behavior in the host hippocampus were comparable between the two age groups. These included the extent of engraftment and migration of graft-derived cells; the degree of differentiation into NeuN+ mature neurons within graft cores; the proficiency for generating GABA+ interneurons or S-100 β + or GFAP+ astrocytes, NG2+ oligodendrocyte precursors, and Olig2+ oligodendrocyte-like cells; and an ability to establish neurogenic niches in non-neurogenic regions, generating new neurons at extended periods after grafting. The only exception is the extent of neuronal differentiation of graft-derived cells that migrated into the neurogenic SGZ-GCL, because greater fractions of graft-derived cells differentiated into

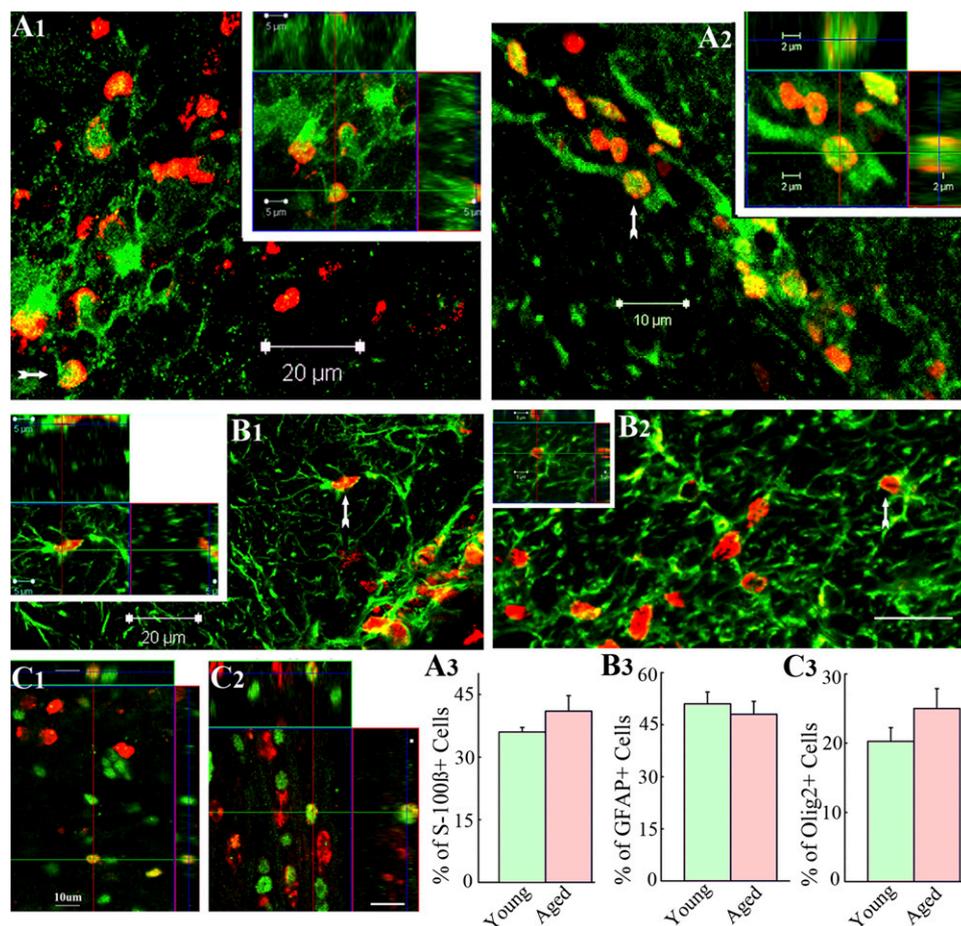


Figure 4. Fractions of cells derived from the subventricular zone-neural stem cell grafts differentiated into S-100β+ (**A1, A2**) and GFAP+ (**B1, B2**) astrocytes and Olig2+ oligodendrocyte-like cells (**C1, C2**) in the intact young (**A1, B1, C1**) and aged (**A2, B2, C2**) hippocampi. (Insets): Orthogonal views of cells indicated by arrows in **A1, A2, B1, and B2**. Scale bar = 20 μm (**A1, B1, B2**) and 10 μm (**A2, C1, C2**). Bar charts show percentages of graft-derived cells that differentiated into S-100β+ astrocytes (**A3**), GFAP+ astrocytes (**B3**), and Olig2+ oligodendrocyte-like cells (**C3**) in the young and aged hippocampi. Abbreviation: GFAP, glial fibrillary acidic protein.

neurons in the young hippocampus compared with the aged hippocampus. These results underscore that advanced age of the host at the time of grafting has no significant adverse effects on engraftment, migration, and differentiation of grafted SVZ-NSCs in the intact hippocampus.

The overall engraftment of graft-derived cells was equivalent to 240%–262% of injected cells in the present study, considerably greater than the ~116% engraftment observed for SVZ-NSC grafts placed into the young hippocampus shortly after injury [30]. This suggests that grafted SVZ-NSCs proliferated considerably after grafting into the intact young or the aged hippocampus. Another study using grafts of NSCs from the fetal hippocampus showed poor engraftment in the injured aged hippocampus [45]. The discrepancy in the extent of engraftment between the previous studies and the present study likely reflects a greater level of inflammation in the injured hippocampus at the time of grafting compared with no inflammation in the intact young hippocampus and relatively milder inflammation in the intact aged hippocampus, because greatly elevated inflammation can interfere with NSC graft cell survival [46]. It is also noteworthy that the extent of survival of SVZ-NSC graft-derived cells in the present study differed greatly from the results of embryonic day 19 (E19) hippocampal cell grafting into intact young and aged hippocampi

reported previously, in which only 18%–23% of grafted cells survived transplantation [40, 47, 48]. Engraftment of fetal hippocampal cells was also modest after grafting into the lesioned aged hippocampus in the absence of graft augmentation strategies [48, 49], consistent with the poor engraftment of fetal cells transplanted using standard conditions in multiple neurodegenerative disease models, including Parkinson's disease [50]. Differences in engraftment between fetal hippocampal cells and postnatal SVZ-NSCs in the intact young or aged hippocampus reflect dissimilarities in the intrinsic properties between the two types of donor cells. Cells harvested from E19 hippocampus are postmitotic neurons committed to differentiation into hippocampal CA1 or CA3 pyramidal neurons and hence are likely more vulnerable to grafting procedure-induced hypoxia and trauma. In contrast, neurosphere cells derived from postnatal SVZ-NSCs are undifferentiated immature cells showing a high propensity for proliferation that can cope with grafting procedure-related hypoxia and trauma better and hence continue their proliferation after grafting. Furthermore, NSCs reside in a hypoxic microenvironment within the brain [51], and physiological hypoxia has been demonstrated to stimulate the survival, growth, proliferation, and multipotency of NSCs [52, 53]. A recent study, in addition, has demonstrated that hypoxia upregulates the calcium-activated calcineurin-NFATc4 pathway in

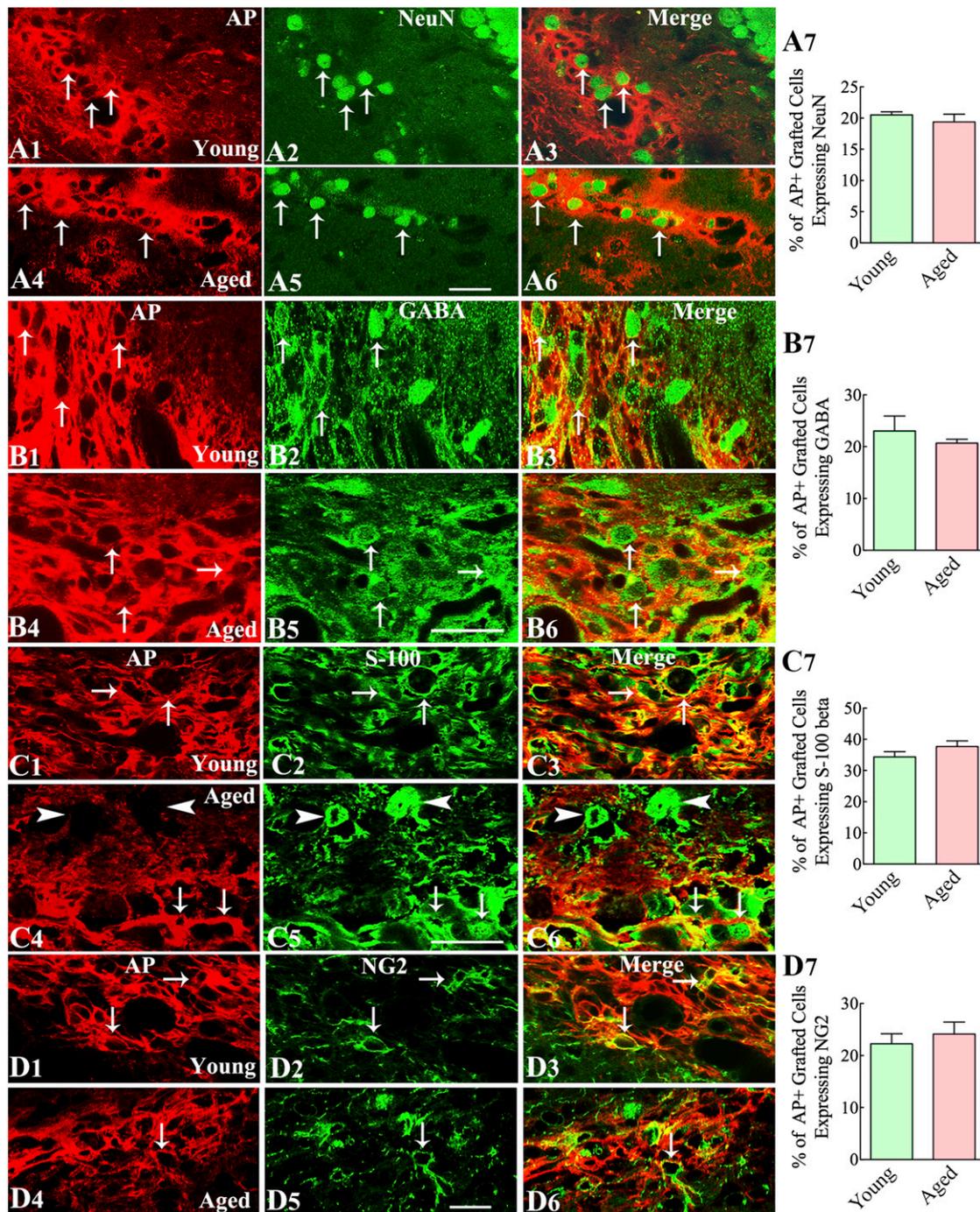


Figure 5. Differentiation of cells derived from the subventricular zone-neural stem cell grafts into neurons and glia in the young and aged hippocampus, as revealed through dual immunofluorescence for AP (graft cell marker) and neural antigens (arrows). (A1–A6): AP+ graft-derived cells that have differentiated into NeuN-expressing neurons in a young hippocampus (A1–A3) and an aged hippocampus (A4–A6). (B1–B6): AP+ graft-derived cells that have differentiated into GABA-expressing interneurons in a young hippocampus (B1–B3) and an aged hippocampus (B4–B6). (C1–C6): AP+ graft-derived cells that have differentiated into S-100β-expressing mature astrocytes in a young hippocampus (C1–C3) and an aged hippocampus (C4–C6). Arrowheads in (C4–C6) denote S-100β+ host astrocytes. (D1–D6): AP+ graft-derived cells that have differentiated into NG2-expressing oligodendrocyte precursor cells in a young hippocampus (D1–D3) and an aged hippocampus (D4–D6). Scale bars = 50 μm (A5, B5, C5, D5). Bar charts compare percentages of NeuN+ neurons (A7), GABA+ interneurons (B7), S-100β+ astrocytes (C7), and NG2+ oligodendrocyte precursors (D7) among graft-derived AP+ cells between the two age groups. Abbreviations: AP, alkaline phosphatase; GABA, γ-aminobutyric acid; NeuN, neuron-specific nuclear antigen.

NSCs, which stimulates their self-renewal and proliferation [51]. Thus, the robust engraftment of NSC-derived cells in the present study compared with the poor engraftment of transplanted fetal

cells reported previously is likely related to the grafting procedure-related hypoxia and propensity of donor SVZ-NSCs for proliferation and multipotent differentiation after grafting.

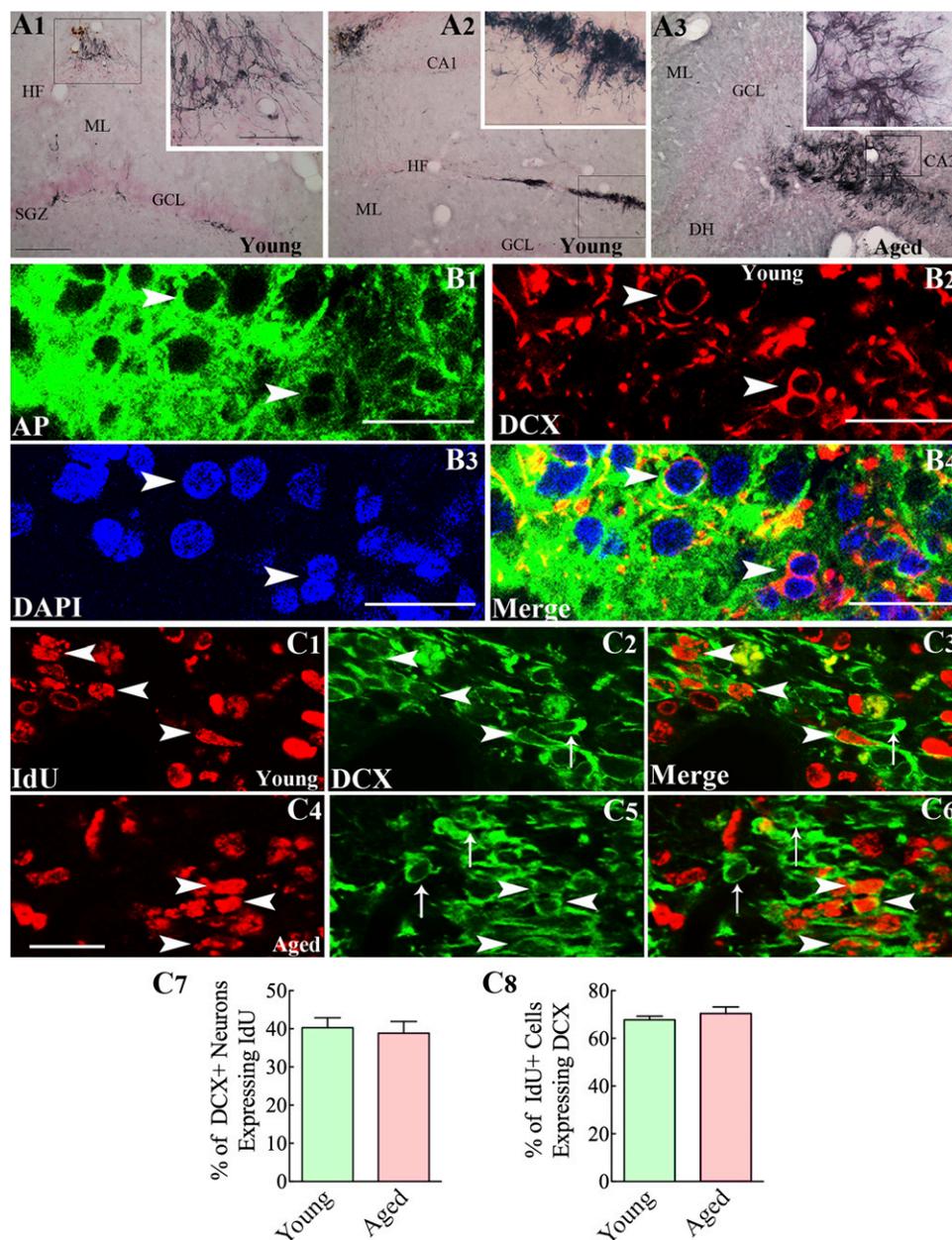


Figure 6. Presence of DCX+ immature neurons in graft cores at 3 months after grafting. **(A1, A2):** Examples of grafts displaying DCX+ immature neurons in intact young hippocampi. **(A3):** An example of a graft in the aged hippocampus exhibiting DCX+ immature neurons. (Insets): Magnified views of graft regions showing the morphology of immature DCX+ neurons. **(B1–B4):** DCX-expressing neurons among graft-derived cells expressing AP. Note that a fraction of AP+ graft-derived cells express DCX (arrowheads). **(C1–C6):** IdU-positive newly born cells expressing DCX within graft cores (arrowheads) located in a young hippocampus **(C1–C3)** and an aged hippocampus **(C4–C6)**. Arrows denote DCX+ neurons that lack IdU. Bar charts compare the percentage of DCX+ neurons that express IdU **(C7)** and the percentage of IdU+ cells that express DCX **(C8)** in grafts located in the young and aged hippocampi. Scale bars = 200 μ m **(A1, B1, C1)**, 50 μ m (insets), and 50 μ m **(B1–C6)**. Abbreviations: AP, alkaline phosphatase; DAPI, 4',6-diamidino-2-phenylindole; DCX, doublecortin; GCL, granule cell layer; HF, hippocampal fissure; IdU, iododeoxyuridine; ML, molecular layer; SGZ, subgranular zone.

The extent of migration of graft-derived cells in the young or aged hippocampus was pervasive in the present study, which was evidenced through engrafting of transplant-derived cells in virtually all layers of the hippocampus. This behavior differs from the fetal hippocampal cell grafts, which have been reported to stay mostly as clusters at the location of grafting [40, 47–49]. The discrepancy in migratory behavior between the two donor cell types again reflects the state of lineage commitment of donor cells. First, E19 hippocampal cells are destined to become CA1 and CA3 pyramidal neurons and have finished their short migration in the

hippocampal primordium at the time of harvesting [40, 47]. In contrast, postnatal SVZ-NSC-derived cells are immature proliferating cells known to display long-distance migration into the olfactory bulb along the rostral migratory stream *in vivo* [2]. Second, E19 hippocampal cells mostly differentiate into neurons after grafting, but SVZ-NSC-derived cells differentiate partially into neurons and predominantly into glia after transplantation. Glial cells typically display a higher propensity for migration than neurons after grafting [54], which could have also contributed toward the excellent migration of the SVZ-NSC graft-derived cells observed in the present study.

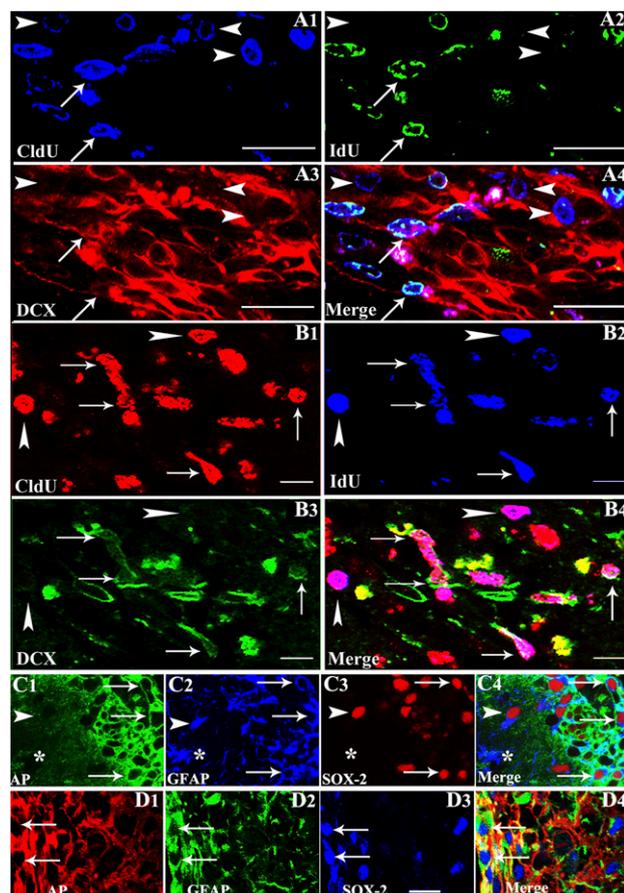


Figure 7. DCX-expressing neurons among graft-derived cells expressing CldU and IdU. **(A1–A4):** Generation of new DCX+ neurons from graft-derived cells in the young **(A1–A4)** and aged **(B1–B4)** hippocampi. Note that a fraction of proliferating cells (IdU+ cells; arrows in **A2** and **B2**) among CldU+ graft cell population (arrows in **A1** and **B1**) express DCX (arrows in **A3** and **B3**), implying that these DCX+ neurons were generated from graft-derived cells during IdU administration (i.e., 1 month after grafting). **(A1–A4):** CldU+ graft-derived cells that lack IdU indicated by arrowheads (i.e., nonproliferating graft-derived cells). **(B1–B4):** CldU and IdU+ cells that lack DCX indicated by arrowheads (i.e., newly born cells that did not differentiate into DCX+ neurons). **(C1–C4):** Graft cores contain putative neural stem cells (NSCs). Note the presence of putative NSCs (triple-labeled cells indicated by arrows in **C1–D4**) expressing AP (graft cell marker; **C1, D1**), GFAP (NSC marker; **C2, D2**), and Sox-2 (another NSC marker; **C3, D3**). **(C1–C4):** Host cells expressing GFAP and Sox-2 (arrowheads). Asterisks indicate examples of a host astrocyte that expresses GFAP but not Sox-2. Scale bars = 50 μm (**A1–B4**) and 20 μm (**C1–C4**). Abbreviations: AP, alkaline phosphatase; CldU, chlorodeoxyuridine; DCX, doublecortin; GFAP, glial fibrillary acidic protein; IdU, iododeoxyuridine.

Comparable differentiation of SVZ-NSC graft-derived cells into NeuN+ mature neurons (within graft cores), GABA+ interneurons, S-100 β + and GFAP+ astrocytes, NG2+ oligodendrocyte precursors, and Olig2+ oligodendrocyte-like cells was observed between the young and aged hippocampus. This was evident from analyses of graft-derived cells using either a label (BrdU) or a transgene (AP) marker. These results suggest that the favorable response of the intact host hippocampus to support the survival, proliferation, and differentiation of SVZ-NSC graft-derived cells remains steady with aging. This is somewhat surprising because aging induces multiple adverse changes in the brain. These include decreased levels of critical neurotrophic factors that are known to support the survival, proliferation, and differentiation of grafted cells [55–58], delayed vascularization of

grafts, and decreased permissiveness for graft axon growth and synaptogenesis [59]. Furthermore, the aged brain displays increased oxidative stress [60, 61], decreased microvasculature and cerebral blood flow [62], and a chronic low level of inflammation in the form of hypertrophied astrocytes and activated microglia [63, 64]. Thus, it is possible that the intrinsic properties of SVZ-NSCs, particularly their ability to thrive in hypoxic conditions, support their engraftment, proliferation, and neuronal and glial differentiation in the aged hippocampus to levels seen in the intact young hippocampus. However, one exception to this rule is neuronal differentiation of SVZ-NSC graft-derived cells that migrated into the neurogenic SGZ-GCL, where a greater percentage of graft-derived cells differentiated into neurons in the young hippocampus than in the aged hippocampus. This discrepancy might be related to specific and drastic age-related changes occurring in the neurogenic dentate gyrus, which could include greatly declined FGF-2 and brain-derived neurotrophic factor signaling [57, 65–68], altered expression of genes that promote neuronal differentiation [69], and considerable microglial activation associated with increased levels of reactive oxygen species and proinflammatory cytokines [70].

Another interesting aspect of the present study is that the grafted SVZ-NSCs showed an ability to establish neurogenic niches in non-neurogenic regions of both young and aged hippocampi. This was evidenced through derivation of DCX-expressing new, immature neurons from graft-derived cells within graft cores located in non-neurogenic regions such as the hippocampal fissure, CA3 subfield, or the CA1 stratum radiatum even at 3 months after grafting. Sequential labeling with CldU (at the time of grafting) and IdU (1 month after grafting) further confirmed that the immature DCX+ neurons found within graft cores were generated from graft-derived cells in both young and aged hippocampi. Because the host age did not influence this phenomenon, it is likely that the intrinsic property of SVZ-NSCs promoted the establishment of neurogenic niches. This possibility is also supported by observations that grafting of NSCs from the postnatal hippocampus into a young or aged hippocampus does not result in the generation of DCX+ immature neurons from graft cores at 3 months after grafting [71]. However, it remains to be determined whether this is an exclusive feature of SVZ-NSC grafts or NSC grafts derived from other sources such as human embryonic stem cells (hESCs) and human induced pluripotent stem cells (hiPSCs) would also exhibit this property. Because neurosphere cells expanded from SVZ-NSCs used for grafting comprised a variety of cell types, including multipotent type 1 radial glia-like cells, type 2 transient amplifying cells, and DCX-expressing immature neurons, the type of cell that promoted the development of neurogenic niche is unknown. Based on intrinsic properties, it is likely that type 1 radial glial cells within the grafts promoted neurogenic niche development by their ability for self-renewal and to generate highly proliferating type 2 transient amplifying cells. Characterization with markers such as AP, GFAP, and Sox-2 did reveal that putative NSCs derived from grafts persist within graft cores. Nonetheless, additional studies are needed in the future for the identification of the specific cell type that will promote the establishment of neurogenic niches with definitive markers of NSCs and intermediate progenitors and whether such niches can be established in lesioned brain areas.

CONCLUSION

The present results demonstrate that advanced age of the host at the time of grafting has no major adverse effects on engraftment,

migration, and differentiation of grafted SVZ-NSCs in the intact hippocampus, as both young and aged hippocampi promoted robust engraftment, migration, and differentiation of SVZ-NSC graft-derived cells. Furthermore, SVZ-NSC grafts showed ability for establishing neurogenic niches in non-neurogenic regions and generating new neurons for extended periods after grafting. This phenomenon is beneficial if these niches can continuously generate new neurons and glia in the grafted hippocampus, as newly generated neurons and glia are expected to improve not only the microenvironment but also the plasticity and function of the aged hippocampus. Overall, these results have significance because the potential application of NSC grafting for treatment of neurodegenerative disorders at early stages of disease progression and age-related impairments would mostly involve aged persons as recipients. Nonetheless, additional studies are needed to determine the functional implications of SVZ-NSC grafting into the aged hippocampus and whether NSCs from other sources such as hESCs and hiPSCs would exhibit similar behavior.

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AUTHOR CONTRIBUTIONS

A.K.S.: conception and design, collection and/or assembly of data, data analysis and interpretation, composite figure preparation, manuscript writing, financial support; B.H.: conception and design, collection and/or assembly of data, data analysis and interpretation, individual figure preparation, manuscript writing.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors indicated no potential conflicts of interest.

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