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HETEROTOPICALLY TRANSPLANTED CVO NEURAL STEM CELLS GENERATE NEURONS AND MIGRATE WITH SVZ CELLS IN THE ADULT MOUSE BRAIN

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Abstract

Production of new neurons throughout adulthood has been well characterized in two brain regions, the subventricular zone (SVZ) of the anterolateral ventricle and the subgranular zone (SGZ) of the hippocampus. The neurons produced from these regions arise from neural stem cells (NSCs) found in highly regulated stem cell niches. We recently showed that midline structures called circumventricular organs (CVOs) also contain NSCs capable of neurogenesis and/or astrogliogenesis in vitro and in situ [3]. The present study demonstrates that NSCs derived from two astrogliogenic CVOs, the median eminence and organum vasculosum of the lamina terminalis of the Nestin-GFP mouse, possess the potential to integrate into the SVZ and differentiate into cells with a neuronal phenotype. These NSCs, following expansion and BrdU-labeling in culture and heterotopic transplantation into a region proximal to the SVZ in adult mice, migrate caudally to the SVZ and express early neuronal markers (TUC-4, PSA-NCAM) as they migrate along the rostral migratory stream. CVO-derived BrdU⁺ cells ultimately reach the olfactory bulb where they express early (PSA-NCAM) and mature (NeuN) neuronal markers. Collectively, these data suggest that although NSCs derived from the ME and OVLT CVOs are astrogliogenic in situ, they produce cells phenotypic of neurons in vivo when placed in a neurogenic environment. These findings may have implications for neural repair in the adult brain.

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Keywords

neural stem cells; adult neurogenesis; nestin

Introduction

The subventricular zone (SVZ) of the anterolateral ventricle wall and subgranular zone (SGZ) of the hippocampal dentate gyrus comprise active neurogenic zones in the adult mammalian brain, providing a continuous supply of neurons for the olfactory bulb (OB) and granule cell layer of the hippocampal dentate gyrus, respectively, throughout the life of the organism. In the SVZ, neural stem cells (NSCs) proliferate and give rise to immature neurons that migrate as the rostral migratory stream (RMS) to the granule cell and glomerular cell layers of the OB, where they differentiate into functional interneurons [2,10,11,17]. NSCs of the hippocampal SGZ migrate a short distance to differentiate into functional granule cells of the dentate gyrus [12,16]. A specialized microenvironment, or neurogenic niche, is critical in providing the instructive and regulatory cues that maintain cell proliferation and differentiation in these neurogenic systems [1,6,13].

Many investigators have taken advantage of the SVZ and SGZ neurogenic niche to test the differentiative potential of putative NSC populations via homotopic or heterotopic transplantation [5,7–11,14,15]. In these studies, various techniques, including labeling with thymidine analogs, adenoviral vectors, and promoter-driven transgenes, are employed to label donor cells, which are then engrafted into the SGZ or SVZ–RMS–OB host site. Interestingly, using this method, cells characteristically non-neurogenic in situ (adult spinal cord progenitors and SVZ ependymal cells) have been shown to undergo neuronal differentiation when transplanted into the SGZ or SVZ [5,14]. Further, these cells migrate and differentiate in a similar fashion to NSCs of the host site.

In our previous work, we showed that in the adult rat and nestin-GFP mouse, midline structures that line the third and fourth ventricles, known collectively as the circumventricular organs, contain cells with NSC characteristics [3]. These cells express NSC markers (nestin, GFAP, vimentin, Sox2), proliferate as neurospheres, and differentiate into neurons, astrocytes, and oligodendrocytes in vitro. In vivo, proliferating cells of intact CVOs differentiate into cells phenotypic of neurons and/or glia; more specifically, the subfornical organ (SFO), organum vasculosum of the lamina terminalis (OVLT), and median eminence (ME) undergo constitutive astroglialogenesis, while cells of the area postrema (AP) undergo constitutive neurogenesis and astroglialogenesis.

We conducted the present study to determine whether CVO NSCs from the adult nestin-GFP mouse could differentiate into cells with a neuronal phenotype when heterotopically transplanted into the instructive environment of the SVZ niche. We will show that BrdU-labeled CVO NSCs derived from the ME and OVLT, regions that are normally astroglialogenic but not neurogenic in situ, can integrate into the SVZ–RMS–OB pathway and differentiate into cells phenotypic of early and mature neurons. These findings confirm our previous results in culture that CVO NSCs are not lineage restricted and demonstrate that CVO cells can generate neurons in vivo when exposed to the appropriate environmental cues which may be important for neural repair and regeneration in the adult brain.

Materials and Methods

All experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals and under approval of Thomas Jefferson University's Institutional Animal Care and Use Committee.

Cell culture

CVO or SVZ tissue from 8–14 week-old transgenic nestin-GFP male and female mice (16 animals per dissection) was visualized under a fluorescent dissection microscope and GFP⁺ tissue was dissected from the ME, OVLT, and SVZ regions as described previously¹. Cells were dissociated in 1 mg/ml papain (Roche, Indianapolis, IN) in HBSS-CMF (Mediatech, Herndon, VA) and plated as suspension culture in NeuroCult NSC Basal Medium with added NeuroCult NSC Proliferation Supplement for mouse (STEMCELL Technologies, Vancouver, BC, Canada). Cultures were supplemented with 20 ng/ml bFGF (R&D, Minneapolis, MN), 20 ng/ml EGF (R&D), and 0.36 units/ml heparin sodium (Abraxis, Schaumburg, IL). By using cells derived from nestin-GFP mice, it was possible to monitor the neural progenitor pool as cells were expanding in culture and select out any contaminating non-green spheres before transplant. To label proliferating cells, 10 μ M BrdU (Fisher Scientific, Fair Lawn, NJ) in basal medium was added every other day for the duration of the culture. There was no evidence of BrdU-induced changes in mitotic rate or cell senescence over time. Cultures were passaged once weekly by trituration with a P200 pipette until dissociation of neurospheres was achieved. After two passages, spheres from three dissections were pooled and grown to approximately 100–200 micron diameter, washed twice with PBS, and resuspended in 3–4 μ l HBSS with 3% glucose for transplantation.

Transplantation

Nine adult male WT (nestin-GFP⁻) mice (8–14 weeks of age) received nestin-GFP⁺ cell transplants (n=4 transplanted with ME donor cells, n=2 transplanted with OVLT donor cells, n=3 transplanted with SVZ donor cells). Animals were anesthetized using isoflurane to effect (1.5–2.5%), and spheres which had been gently triturated into small aggregates of donor cells (approximately 200,000 cells per transplant) were delivered stereotactically with a Hamilton syringe into the following coordinates: AP +0.3, ML +1.5, DV -2.25 (from top of brain).

BrdU Staining/Immunocytochemistry

Ten (OVLT) or 14 days (ME, SVZ) after the transplantation procedure adult male WT (nestin-GFP⁻) mice were deeply anesthetized with a cocktail of ketamine (100 mg/kg) and xylazine (5 mg/kg, mice) and perfused with cold (4°C) periodatylsine-paraformaldehyde (4%). Brain sections were cut at 14 μ m thickness on a cryostat, and sections were incubated with primary antibodies in a 0.01 M PBS solution containing 0.3% Triton $\times$ 100 and 2% normal donkey serum (NDS). After incubation overnight at 4°C, sections were washed five times for ten minutes each with a 0.01 M PBS solution at room temperature. Secondary immunofluorescent antibodies were incubated for one hour at room temperature in 0.01 M PBS solution containing 2% NDS. The nuclear dye Hoechst 33258 (Invitrogen, 1 mg/ml) was added to secondary antibody solution for nuclear staining. Sections were then washed, covered and examined on an Olympus IX2-UCB with a Sensicam digital camera system (Cooke) and Slidebook imaging software (Intelligent Imaging Innovations).

For BrdU staining, tissue sections were washed with 0.01 M PBS and then treated with 2M HCl for 30 minutes at room temperature. Sections were then washed with 0.01 M PBS followed by incubation for 30 minutes at room temperature with 0.1 M sodium tetraborate decahydrate. Sections were then washed with 0.01 M PBS and processed for immunocytochemistry as above.

Antibodies—Sections were incubated with primary antibodies Accurate Chemical rat anti-bromodeoxyuridine (Westbury, NY, OBT0030, 1:100), Millipore rabbit anti-TUC-4 (Billerica, MA, AB5454, 1:100), Millipore chicken anti-GFP (AB16901, 1:200), DSHB mouse anti-PSA-NCAM supernatant (Iowa City, Iowa, 5A5; 1:2), DAKO rabbit anti-GFAP (Carpinteria, CA, Z0334, 1:1000), Sigma rabbit anti-GABA (St. Louis, MO, A2052, 1:2000), Pel-Freez rabbit anti-tyrosine hydroxylase (Rogers, AR, P40101-0, 1:100) or Millipore mouse anti-NeuN (MAB377, 1:100). All secondary antibodies were Alexa Fluor antibodies from Invitrogen: donkey anti-rabbit 488, 1:200; donkey anti-mouse 488, 1:200; donkey anti-rat 594, 1:300; and goat anti-chicken 488, 1:200.

In colocalization studies, the treatment of tissue with HCl during the BrdU staining process often quenched endogenous GFP fluorescence. Therefore, an antibody to GFP was used to increase sensitivity during staining. For confocal analysis, immunofluorescent double-labelings (BrdU/TUC-4 and BrdU/PSA-NCAM) were analyzed using a laser scanning confocal microscope (Olympus Fluoview, Olympus America Inc., Center Valley, PA) with an automatically optimized pinhole size and 0.6 μ m thickness of the calculated optical section. Each section was analyzed with the 40 $\times$ objective and scanned successively with helium/neon (for Alexa 594 fluorescence) and the argon/krypton (for Alexa 488 fluorescence) lasers over 15–20 focal planes with a 512 $\times$ 512 pixel resolution.

Results

CVO Cells Survive Heterotopic Transplantation and Migrate to SVZ

To assess CVO-derived NSC behavior after heterotopic transplantation into the SVZ region, donor cells were first expanded in culture. Cells from the ME, OVLT, or, as a positive control, from the known neurogenic SVZ region were microdissected from adult nestin-GFP mice, grown in culture as GFP⁺ neurospheres, and treated with 10 μ M BrdU every other day to label most proliferating cells. After at least 14 days in vitro, spheres were harvested and delivered stereotactically with a Hamilton syringe to a location proximal to the SVZ in WT (GFP⁻) littermates (Figure 1A, I). Mice were perfused at 10 (OVLT) or 14 (ME or SVZ) days post-transplant, and tissue was processed for immunocytochemistry. For ease of understanding the putative pathway of cell migration, Figure 1A illustrates the injection site of CVO-derived donor cells (I), the SVZ (II), the RMS (III), and the OB (IV). These identifiers (I–IV) will be used to clarify the host location displayed in each figure. As shown in Figure 1, BrdU⁺ cells were located at the transplant site (I); shown here are BrdU-labeled donor cells from the ME (ME TX, B), OVLT (OVLT TX, D), and SVZ (SVZ TX, F). Although the brain sections shown here did not contain cells with residual endogenous GFP fluorescence, in other animals, faint GFP⁺ cells of varying number were observed at the transplant site (see Fig. 1B, D, F insets). Interestingly, BrdU⁺ transplant cells appeared to have migrated caudally from the implantation site to reach the SVZ (Figure 1A, II), as shown in panels C, E, and G of Figure 1.

When the numbers of BrdU⁺ cells from the various donor regions were quantified, interestingly, more labeled cells were found in the SVZ transplant site (and in the RMS and OB) when cells were derived from the SVZ (82 ± 12) than the ME (30.8 ± 3.2) or OVLT (31.4 ± 10.4).

CVO-derived Cells Travel In Host RMS

We next stained host animal sections for BrdU and GFP and the early neuronal markers PSA-NCAM and TUC-4 to assess whether transplant cells could be visualized traveling in the RMS (Figure 1A, III). As expected, by 10–14 days post-transplantation, cells in the RMS no longer expressed nestin (ie. GFP) but could be tracked by BrdU and other early markers of neuronal differentiation. Thus, as shown in Figure 2, BrdU⁺PSA-NCAM⁺ and BrdU⁺TUC-4⁺ cells were

located in the RMS of mice transplanted with cells from the ME (A–C), OVLT (D–F), and SVZ (G–I). When quantified (Table 1), the proportion of Tuc4⁺ BrdU-labeled cells in the RMS was highest for the SVZ (69.4%) compared with the OVLT (39.6%) and ME (16.4%) donor cells.

Because the RMS consists of densely packed cells, we confirmed co-expression of BrdU and neuronal markers PSA-NCAM or TUC-4 using confocal microscopy. Shown are the XZ plane (yellow lines, bottom panels of C, F, I) and YZ plane (pink lines, right panels of C, F, I) through an ME-derived BrdU⁺PSA-NCAM⁺ cell (C) an OVLT-derived BrdU⁺TUC-4⁺ cell (F), and through an SVZ-derived BrdU⁺PSA-NCAM⁺ cell (I) in the RMS. These cells were confined to the RMS path and not located in adjacent parenchyma. Some transplanted BrdU⁺ CVO and SVZ cells migrating in the RMS did not co-label with either TUC-4 or PSA-NCAM and their phenotype remains unknown.

CVO-derived Cells Migrate to Host OB and Express Neuronal Markers

Finally, using double-label immunocytochemistry, we assessed whether BrdU labeled CVO cells had migrated to the host OB (Figure 1A, IV). Although BrdU⁺ cells survived in all grafts, cells successfully migrated to reach the OB in 1 of 3 SVZ transplants, 1 of 2 OVLT transplants, and 2 of 4 ME transplants. Consistent with their time-dependent movement to the target region, in all cases, more BrdU⁺ cells were observed in the OB at later than earlier post-transplant times (14 vs. 10 days). As shown in Figure 3, BrdU⁺ nuclei from these animals were indeed observed in the OB in GFP[−] (A, D, G) and GFAP[−] (B, E, H) cells, suggesting that these were neither stem cells nor astrocytes. Instead, like BrdU-labeled cells derived from the positive control SVZ region (H), BrdU⁺ cells from the ME and OVLT transplants co-expressed the neuronal marker NeuN (C, F insets) at 10–14 days post-transplant.

When quantified following their arrival in the OB, the vast majority of cells from all three donor regions co-expressed neural (Tuc4, NeuN) as compared to glial (GFAP) markers (Table 1). With the exception of OVLT which was examined at an earlier time point (10 vs 14 days post-transplantation), donor (BrdU⁺) neurons in the target OB region expressed more mature (NeuN) than immature (Tuc4) neural markers.

Although donor-derived neurons were located at the termination of the RMS and in the granule cell layer, they did not express the granule cell neurotransmitter GABA (data not shown). BrdU⁺ donor cells were also negative for the enzyme tyrosine hydroxylase, which is normally expressed in SVZ-derived periglomerular interneurons (data not shown).

Discussion

This study demonstrates that NSCs from two CVOs, the ME and OVLT, possess the potential to integrate into the most active adult neurogenic site of the mammalian brain and to differentiate into cells with a neuronal phenotype. These NSCs, following expansion and BrdU-labeling in culture and heterotopic transplantation into a region proximal to the SVZ of adult WT littermates, appeared to migrate caudally to the SVZ where many cells co-expressed early neuronal markers (TUC-4, PSA-NCAM) as they migrated along the RMS. In many cases, CVO-derived BrdU⁺ cells ultimately reached their target region, the OB, where they co-expressed early (PSA-NCAM) or mature (NeuN) neuronal markers. The most likely explanation for why BrdU⁺ cells were not detected in the OB in all cases is the improper placement of donor cells in the host brain, as SVZ cell migration has been reported with homotopic transplantation [7,9,11]. Collectively, these data suggest that NSCs derived from the ME and OVLT are not lineage restricted and can produce neurons *in vivo* when placed in a neurogenic environment, thus having possible implications for neural repair of the adult brain.

In this study, we used BrdU to distinguish our donor cells from endogenous progenitors in the SVZ. It is important to note that although thymidine analogs are used as a standard in the field to label proliferating cells, it has been reported in one study that BrdU derived from dead cells can be taken up by host progenitors[4]. This is in disagreement with several reports claiming that endogenous cells do not take up BrdU from damaged or dead cells [7,13,14]. As a cautionary measure, the remote possibility should be considered that BrdU may have labeled not only donor cells but also endogenous cells in the present study. A transgenic reporter line that allows for cell progeny tracing will be needed in place of BrdU-labeling to entirely rule out this possibility.

Normally SVZ NSCs give rise to functional interneurons of the glomerular layer and GABA-ergic interneurons of the granule cell layer in the OB. Notably, we observed BrdU-labeled NeuN⁺ cells in the OB granule cell layer of the host animal transplanted with ME donor cells and perfused 14 days post-transplantation. These BrdU⁺ cells in the granule cell layer did not express GABA, however, regardless of whether they were ME or SVZ derived. It is likely that a longer time period between transplantation and perfusion is necessary to develop the mature neurotransmitter phenotype and functional status of donor-derived cells in the OB.

The present study demonstrates that NSCs from the ME and OVLT, both astroglialogenic but not neurogenic in situ, can be instructed to produce cells phenotypic of neurons in vivo. This suggests that the CVO microenvironment may restrict NSC differentiation toward a glial lineage. Future studies are needed to elucidate the molecular signals that instruct CVO NSC fate in situ. In addition, an examination of the CVO NSC microenvironment could also aid in our understanding of the NSC niche and its importance in cell fate determination.

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Figure 1.

Heterotopic transplantation and migration of CVO-derived cells. Shown in Figure 1A in schematic depiction are the transplantation site of CVO cells (I), and the putative pathway of CVO-derived cell migration: the subventricular zone (SVZ, II), the rostral migratory stream (RMS, III, blue), and the olfactory bulb (OB, IV). Following BrdU-labeling of nestin-GFP⁺ CVO and SVZ cells in culture, BrdU⁺ cells were located at the transplant site (I); shown here are immunocytochemically stained donor cells from the ME (ME TX, B) and OVLT (OVLT TX, D) CVO regions, and donor cells from the known neurogenic SVZ region (SVZ TX, F). These cells were no longer GFP⁺ (yellow likely represents lipofuscin granules from degenerated donor cells) although GFP⁺BrdU⁺ nuclei (insets) were identified at the ME, OVLT

and SVZ transplant sites in other sections. Interestingly, BrdU⁺ cells appeared to have migrated dorsally to reach the SVZ (Figure 1A, II), as shown in panels C, E, and G of Figure 1. LV: lateral ventricle. Calibration bar = 50 μ m

Figure 2.

CVO-derived cell migration in the rostral migratory stream (RMS). Shown are immunocytochemically stained images from the RMS (site III as depicted in Figure 1A) of host mice. BrdU⁺PSA-NCAM⁺ and BrdU⁺TUC-4⁺ cells (arrows) are shown in the RMS of mice transplanted with ME cells (A–C insets), OVLT cells (D–F insets), and control SVZ cells (G–I insets), indicating differentiation toward a neuronal fate and migration of transplanted donor cells. Co-labeling of BrdU with TUC-4 or PSA-NCAM was confirmed with confocal microscopy (C, F, I). Shown are the XZ plane (yellow lines, bottom panels of C, F, I) and YZ plane (pink lines, right panels of C, F, I) through an ME-derived BrdU⁺PSA-NCAM⁺ cell 14 days post-transplant (C), an OVLT-derived BrdU⁺TUC-4⁺ cell 10 days post-transplant (F), and an SVZ-derived BrdU⁺PSA-NCAM⁺ cell (I) in the RMS at 14 days post-transplant. Calibration bar = 25 μ m

Figure 3.

CVO-derived cell migration to the olfactory bulb (OB). Shown are images from the OB (site IV as depicted in Figure 1A) of host mice. BrdU⁺ nuclei from host animals transplanted with ME or OVLT cells are shown in the OB and are nestin-GFP⁻ (A, D) and GFAP⁻ (B, E), indicating that these are neither stem cells nor astrocytes. Instead, like BrdU⁺ cells derived from the control SVZ region (H, arrow; inset), BrdU-labeled cells from the ME and OVLT transplants co-expressed the neuronal markers NeuN (C, F arrow; inset) at 10–14 days post-transplant. These cells were located at the termination of the RMS and in the granule cell layer of the OB. Calibration bar = 25 μ m

Table 1

Colocalization of neural and glial markers in BrdU-labeled CVO donor cells after transplantation into the brain.

<i>ME donor</i>				
	BrdU ⁺	Tuc4 ⁺ /BrdU ⁺ (% of average BrdU ⁺)	NeuN ⁺ /BrdU ⁺ (% of average BrdU ⁺)	GFAP ⁺ /BrdU ⁺ (% of average BrdU ⁺)
SVZ	30.8±3.2	7.8±1.69 (26±5%)	-----	-----
RMS	32±12.6	16.4±6.63 (51±21%)	-----	-----
OB	15.4±4.0	2.6±1.07 (17±7%)	8.2±2.2 (52±15%)	2.8±1.05 (18±7%)
<i>OVL donor</i>				
SVZ	31.4±10.4	14±3.67 (45±12%)	-----	-----
RMS	54±23.8	39.6±13.9 (73±26%)	-----	-----
OB	11.6±5.9	8.8±4.32 (76±37%)	1.2±0.44 (10±4%)	1.8±0.84 (16±7%)
<i>SVZ donor</i>				
SVZ	82±12	66.2±8.7 (81±11%)	-----	-----
RMS	84.4±17.8	69.4±8.68 (82±10%)	-----	-----
OB	70.4±23	25±8.94 (36±13%)	44.4±13.1 (63±19%)	4.6±2.06 (7±3%)

Values are reported in mean # of cells per 14 µm section ± SEM.