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Heterotopically transplanted CVO neural stem cells generate neurons and migrate with SVZ cells in the adult mouse brain

Lori B. Bennett[a], Jingli Cai[a], Grigori Enikolopov[b], Lorraine Iacovitti[a,*]

[a] Farber Institute for Neurosciences, Department of Neurology, Thomas Jefferson University Medical College, 900 Walnut Street, Philadelphia, PA 19107, United States
[b] Cold Spring Harbor Laboratory, 1 Bungtown Road, Cold Spring Harbor, NY 11724, United States

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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 (Bennett et al. [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.

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 astrogliogenesis, while cells of the area postrema (AP) undergo constitutive neurogenesis and astrogliogenesis.

We conducted the present study to determine whether CVO NSCs from the adult nestin-GFP mouse could differentiate into cells

* Corresponding author at: Farber Institute for Neurosciences, Thomas Jefferson University Medical College, Suite 462-Jefferson Hospital for Neuroscience, 900 Walnut Street, Philadelphia, PA 19107, United States. Tel.: +1 215 955 8118; fax: +1 215 955 2993.
E-mail address: lorraine.iacovitti@jefferson.edu (L. Iacovitti).

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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 astrogliogenic 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.

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.

CVO or SVZ tissue from 8- to 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 a described previously [1]. 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 μM 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 μl HBSS with 3% glucose for 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 isofluorane 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 stereotaxically with a Hamilton syringe into the following coordinates: AP +0.3, ML +1.5, DV −2.25 (from top of brain).

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) periodate–lysine–paraformaldehyde (4%). Brain sections were cut at 14 μm thickness on a cryostat, and sections were incubated with primary antibodies in a 0.01 M PBS solution containing 0.3% Triton X100 and 2% normal donkey serum (NDS). After incubation overnight at 4 °C, sections were washed five times for 10 min each with a 0.01 M PBS solution at room temperature. Secondary immunofluorescent antibodies were incubated for 1 h 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 2 M HCl for 30 min at room temperature. Sections were then washed with 0.01 M PBS followed by incubation for 30 min 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 μm thickness of the calculated optical section. Each section was analyzed with the 40× 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 × 512 pixel resolution.

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 μM BrdU every other day to label most proliferating cells. After at least 14 days in vitro, spheres were harvested and delivered stereotaxically with a Hamilton syringe to a location proximal to the SVZ in WT (GFP$^-$) littermates (Fig. 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, Fig. 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 Fig. 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, and F insets). Interestingly, BrdU$^+$ transplant cells appeared to have migrated caudally from the implantation site to reach the SVZ (Fig. 1A, II), as shown in panels C, E, and G of Fig. 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).

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 (Fig. 1A, III). As expected, by 10–14 days post-transplantation, cells in the RMS no longer expressed nestin (i.e. GFP) but could be tracked by BrdU and other early markers of neuronal differentiation. Thus, as shown in Fig. 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