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Identification of a Sustained Neurogenic Zone at the Dorsal Surface of the Adult Mouse Hippocampus and Its Regulation by the Chemokine SDF-1

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Abstract

We identified a previously unknown neurogenic region at the dorsal surface of the hippocampus; (the “subhippocampal zone,” SHZ) in the adult brain. Using a reporter mouse in which SHZ cells and their progeny could be traced through the expression of EGFP under the control of the CXCR4 chemokine receptor promoter we observed the presence of a pool of EGFP expressing cells migrating in direction of the dentate gyrus (DG), which is maintained throughout adulthood. This population appeared to originate from the SHZ where cells entered a caudal migratory stream (aCMS) that included the fimbria, the meninges and the DG. Deletion of CXCR4 from neural stem cells (NSCs) or neuroinflammation resulted in the appearance of neurons in the DG, which were the result of migration of NSCs from the SHZ. Some of these neurons were ectopically placed. Our observations indicate that the SHZ is a neurogenic zone in the adult brain through migration of NSCs in the aCMS. Regulation of CXCR4 signaling in these cells may be involved in repair of the DG and may also give rise to ectopic granule cells in the DG in the context of neuropathology.

Keywords

adult neurogenesis; dentate gyrus; chemokines; CXCR4; SDF-1

INTRODUCTION

Neurogenesis persists in two main areas of the adult mammalian brain, the neurogenic subventricular zone (SVZ) of the lateral ventricles (LVs), and the subgranular zone (SGZ) of

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the hippocampal dentate gyrus (DG; Zhao et al., 2008). The SVZ, a well studied neurogenic area, harbors a heterogeneous population of adult stem cells and progenitors which include radial astrocyte like cells (also called type B cells or SVZ radial astrocytes), transit amplifying cells (type C cells), and neuroblasts (type A cells). In the adult brain some of radial astrocyte like cells in the SVZ act as neural stem cells (NSCs) capable of generating neuroblasts which migrate along a well-defined route known as the rostral migratory stream toward the olfactory bulb (OB) where they eventually differentiate into a wide variety of inter-neuron subtypes (Luskin, 1993; Peretto et al., 1997; Alvarez-Buylla and Garcia-Verdugo, 2002; Merkle et al., 2014). In the hippocampal DG, a subset of quiescent radial astrocyte like cells in the SGZ act as stem cells that are able to proliferate and generate new neurons throughout life. These cells self-renew and produce rapidly amplifying progenitor cells which express doublecortin (DCX). The DCX-positive cells migrate a short distance within the mature granular cell layer where they begin to express markers of mature neurons (van Praag et al., 2002; Arous et al., 2005; Kee et al., 2007; Zhao et al., 2008; Zhao and Overstreet-Wadiche, 2008).

Chemokines are small secreted proteins that play an important role in leukocyte trafficking and are now also recognized as being important regulatory factors in the development of most tissues including the nervous system (Ransohoff, 2009; Mithal et al., 2012). Stromal cell derived factor-1, (SDF-1, also known as CXCL12) and its receptor CXCR4 regulate the migration and development of stem cells in many tissues including the nervous system (Bagri et al., 2002; Lu et al., 2002; Stumm et al., 2003; Belmadani et al., 2005, 2006; Tran et al., 2007; Li and Ransohoff, 2008; Wang et al., 2011). For example, during the formation of the DG, granule cell progenitors born at different locations of the ventricular zone (VZ) of the dentate primordium (VDP) near the prospective fimbria of the hippocampus, first migrate along a primordial radial glial scaffold (Eckenhoff and Rakic, 1984; Rickmann et al., 1987; Altman and Bayer, 1990; Li et al., 2009) and form a transient subpial proliferative zone that gives rise to the outer shell of the granule cell layer of the DG (Li et al., 2009). This process requires SDF-1/CXCR4 mediated signaling (Bagri et al., 2002; Lu et al., 2002; Li and Pleasure, 2005; Borrell and Marin, 2006; Berger et al., 2007; Lopez-Bendito et al., 2008; Li et al., 2009). During the last week of gestation, it is believed that the pool of subpial precursors shifts into the dentate hilus (hilar dentate matrix) and eventually relocates to the SGZ, which becomes the permanent site of adult hippocampal neurogenesis (Rickmann et al., 1987; Altman and Bayer, 1990; Li et al., 2009). Consequently, the VDP collapses near the fimbria and its remnant becomes part of a restricted area of the medial walls of the LVs. Recently, however, it was shown that NSCs in the ventral hippocampus during late in gestation are the source of the long-lived NSCs of the SGZ in the DG (Li et al., 2013). These embryonic progenitor cells express the chemokine receptor CXCR4 as those in the major neurogenic niches of the adult brain, the SVZ (Ji et al., 2004; Tran et al., 2007; Liu et al., 2008) and the SGZ (Kempermann et al., 2004; Tran et al., 2007). We have now identified a new migratory stream of CXCR4 expressing cells from a previously unrecognized neurogenic zone in the medial walls of the LVs covering the dorsal surface of the hippocampus. These cells first exit the LVs through the fimbria-dentate junction (FDJ) and navigate the hippocampal fimbria as a stream of migratory cells ultimately reaching the meninges. The cells then closely migrate along the meninges in the direction of the DG. As

these cells follow a caudal migratory route in the adult brain we termed this pathway the adult caudal migratory stream (aCMS).

MATERIALS AND METHODS

Sources of Mice

CXCR4-EGFP mice were acquired from GENSAT (Gong et al., 2003). The Nestin-Cre line was a donation from Dr. Anjen Chenn (Northwestern University). The CXCR4^{fl/fl} mouse was a donation from Dr. Yong-rui Zhou (Columbia University). The Rosa26-YFP mouse line was a donation from the Dr. Raj Awatramani at (Northwestern University). SDF1:mRFP mice were generated from our Laboratory by Dr. Hosung Jung as described previously (Jung et al., 2009). CD1 mice were purchased from (Charles River Laboratories).

Generation of Bicolor Mice

SDF1-mRFP/CXCR4-EGFP mice were generated through a standard backcrossing paradigm over the course of two years and mice were used after the 10th generation of backcrossing. Housing, breeding and crossing, as well as research procedures performed were approved by the Northwestern University Institutional Animal Care and Use Committee.

Generation of CXCR4 Conditional Knockout Mice

To achieve CMS-specific knockout of CXCR4, we used the “Cre-Lox” system with a nestin promoter driven Cre. Mice homozygous for the floxed CXCR4 gene (cxcr4^{fl/fl}) mice were crossed with Nestin-Cre mice, which express Cre recombinase in SHZ and CMS cells expressing nestin. CXCR4^{fl/+}, Cre^{fl} and CXCR4^{fl/fl} that resulted were interbred to generate Nestin-Cre conditional knockout cxcr4 animals (cxcr4^{fl/fl}, Cre^{fl}). These mice were screened for germ-line transmission of the Cre and floxed alleles by PCR analysis. To evaluate the effectiveness of this transgene in the removal of cxcr4 itself in nestin-Cre/ cxcr4^{fl/fl} animals, Nestin-Cre conditional cxcr4 mutant mice were backcrossed with Rosa26-YFP to generate cxcr4 ko YFP mice, and YFP cells were sorted by FACS and subjected to PCR analysis and Fura-2 calcium imaging assay. PCR products showed that the expression of an active cxcr4 transcript was greatly reduced in target cells in nestin-Cre/cxcr4^{fl/fl} animals compared to floxed animals. Similarly, when the Fura-2 based assay with YFP FACS sorted cells was used in response to SDF-1, a direct indication of CXCR4 signaling, we observed that CXCR4 signaling was almost completely absent from YFP cells compared to a clear and transient response of similar cells taken from CXCR4 floxed animals.

Brain Sectioning, Imaging, and Image Processing

Animals were anesthetized and fixed in 4% paraformaldehyde (PFA). Brains were removed and postfixed in 4%PFA for 48 h, washed in PBS, and then transverse and sagittal 40 μ m thick sections were cut using a Leica VT 1000S vibratome. Sections were either analyzed directly by confocal microscopy to observe for epifluorescence or prepared for immunostainings. Imaging was performed on the Olympus FluoView FV10i confocal laser scanning microscope (FV10i, Olympus Corporation of America, Center Valley, PA) using 10 $\times$ and 60 $\times$ objectives with the aid of 1–6 optical zoom. Using this new and powerful

machine we had the ability to use a map image mode and observation mode to acquire z-stack images. Image processing and analysis including localization and fluorescence analysis were done using the FV10i accompanying software (Version 02.01c; Olympus), followed by image enhancement using ImageJ or Photoshop CS3.

Immunofluorescence

Immunostaining was performed using free floating 40 μ m-thick sections as was previously described (Belmadani et al., 2006). Briefly, sections were blocked in phosphate buffer containing 0.1% Triton X-100 and incubated overnight at 4 °C with the following primary antibodies: CD45 (1/300, rat, Millipore, CA) and Iba-1 (1/300, rabbit, Wako Chemicals USA, VA) for microglia; CD45 and F4/80 (1/300, rabbit, Santa Cruz Biotechnology) for macrophages; Nestin (1/150, rat, BD Pharmingen, CA) for early neural progenitors, SOX-2 (1/200, rabbit, Millipore, CA) for neuronal stem cells, GFAP (1/300, mouse, Sigma-Aldrich, MO) and BLBP (1/100, rabbit, Millipore) for radial glia, DCX [1:700; Guinea pig, Millipore, CA) for migratory neuroblasts, NeuN (1/300, mouse, Millipore, MA), Prox-1 (1/500, rabbit, Millipore, CA)] for DG granular neurons, calretinin and calbindin (1/250, rabbit, Millipore, MA) for mature DG neurons, laminin (1/100, rabbit, Millipore, CA), vWF (1/100, rabbit, Santa Cruz Biotechnology, CA) for blood vessels. This was followed by incubation with specie-specific secondary antibodies conjugated with fluophores (1/500, Invitrogen, OR) or biotin (1/250) followed by streptavidin conjugated fluophores (1/100, Molecular Probes, OR). The sections were then mounted under glass coverslips with Vectashield antifade reagent with DAPI (Vecta-shield, Vector Laboratories, CA) and imaged with FV10i confocal microscope. When acidic solution (to allow for DNA denaturation) or heat-induced antigen retrieval were required, green fluorescent protein (GFP) antibody (1/200, mouse, Millipore, MA) was also used to better visualize GFP. For neurosphere characterization, different CXCR4 antibodies (1/200, rabbit and goat, Santa Cruz Biotechnologies, CA) were used to verify the expression of CXCR4 by CMS-EGFP NSs in cultures.

Neurosphere Cultures and Calcium Imaging

To generate SHZ NSs, we dissected several hippocampi from 8weeks old tg CXCR4-EGFP mice. A quick examination of each hippocampus (dorsal side) under fluorescent scope showed a thick membrane packed with strongly fluorescent cells (green fluorescence), which formed the medial walls of the LV. We then carefully removed DG from each hippocampus while saving the fimbria and the medial wall of the LV intact. These were cut into small pieces using a tissue shopper, washed 3 times in Hanks balanced salt solution (HBSS), and then digested with 0.1% papain for 40 min at 37 °C. Tissue pieces were then washed twice with HBSS, once with medium (DMEM-F12, 3:1, 1% penicillin/streptomycin containing 5% bovine serum), and twice with serum-free medium. Hippocampal pieces were then mechanically dissociated in medium with the aid of needles of the respective sizes 18, 21, and 27 and the suspension poured through a 40 μ m cell strainer (Falcon, Franklin Lakes, NJ). Dissociated cells were centrifuged at 168 g and either directly processed for fura-2 based calcium imaging assay to test for functional expression of CXCR4 (Belmadani et al., 2001) or sorted by FACS. Next they were incubated in a proliferation medium containing DMEM/F12 (3:1) with B-27 (Gibco-BRL), supplemented with basic fibroblast growth factor (bFGF; 20 ng/ml, R&D Systems, Minneapolis, MN), the Epidermal growth factor (EGF; 20

ng/ml, R&D Systems) to examine for self-renewal using neurosphere formation. After 7–15 days, sometimes 21 days later in cultures, obtained primary NSs were processed for Ca imaging or subjected to immunostaining or migration assay. In some instance, SDF-1 (10 ng/ml) was added to test for its mitogenic effects and to help NSs formation.

Transwell Migration Assay (TMA)

To assess for chemotaxis of SHZ cells, we used Cell Culture Insert with transparent PET membrane (Becton Dickinson, 8 μ m, 12 well format). This assay allowed us to assess the migration of cells in response to a gradient of a chemoattractant between the top of the membrane and underneath the membrane of the insert. To perform this assay, we prepared a suspension of CXCR4-EGFP cells dissociated from either a fresh SHZ (SHZ-CXCR4-EGFP cells) or SHZ NSs that we supplemented with 2.5% matrigel and plated on the top of the membrane pre-coated with matrigel (5%). After 6 h incubation, we added SDF-1 (50 nM) or SDF-1 (50nM) + AMD3100 (10 μ M), a specific CXCR4 antagonist underneath the membrane for an additional 45 min. To determine the migration index, membranes were fixed in 4% PFA and the top of the membranes were cleaned with a cotton swab. Membranes were then cut and mounted on a coverslip for microscopic imaging. They were imaged under the FV10i confocal microscope and cells that were migrated to the bottom of the membrane were counted in 6 randomly selected fields. The migration index is determined as the number of cells that migrated into the bottom of the membrane in each treated group compared with control group. Statistical differences were assessed by student *t*-test at $P < 0.05$. In some experiment, post hoc Fura-2 calcium imaging was processed on migrated cells after the first 6 h incubation to test for functional chemokine receptors.

BrdU Labeling

To label slowly dividing SHZ cells a daily intraperitoneal Bromodeoxyuridine (BrdU; Sigma-Aldrich, 33.33 mg/kg) injections were given into CXCR4-EGFP mice, twice a day for 6 days, and mice were killed 24 h later after the last injection. To label rapidly dividing SHZ cells and their descendants, mice were injected once and killed 2 h later. Whole-mount fixed preparations or free floating fixed sections were then incubated in 1N HCl for 30 min at 45 °C and neutralized in PBS before immunostainings for Brdu (1/500, sheep, Fitzgerald Industries International, MA) and Ki67 (1/100, rabbit, BD Pharmingen, CA) for proliferating cells exiting the cell cycle.

Retrovirus-Mediated SHZ Labeling and Analysis of aCMS Cells

To make sure that the cells observed within the aCMS pathway originate in a specific region of the LV, we labeled cells of the LV by intraventricular injection of retrovirus expressing mCherry. The mCherry retrovirus is expected to label dividing and quiescent cells and provide stable long term expression. This allowed us to mark all dividing cells of the SHZ niche and further explore their migratory capacity and neuronal development in subsequent studies. To do this, mice were anesthetized with intraperitoneal injection of avertin (33 mg/kg), placed on a stereotaxic device (Stoelting, IL) and injected with an Hamilton syringe with 26 gauge needles containing 2 μ l solution of mCherry retrovirus (gift from Dr John Kessler, Northwestern University) into the LV at the following coordinates: 1.94 mm

posterior to Bregma, 2.8 mm lateral to the midline and 2.4–2.8 mm ventral from the pia. At the end of the experiments, mice were anesthetized, and brains were subjected to vibratome sectioning. Following sectioning of the brains, expression patterns of mCherry cells in the SHZ and along their migratory pathway within the aCMS was documented.

HIV-1-Mediated Neuroinflammation and Neurodegeneration in the DG of the Adult Hippocampus

We used an engineered recombinant HIV-1 viruses (HXB2), replication-deficient of the X4-tropic variants referred to as HXB2 pseudotyped HIV-luc viruses. They were generated by cotransfecting DNA encoding HXB2 HIV envelope plasmids (pNL4-3-Luc-R1-Env) along with Envdeficient HIV vector (pNL4-3-Luc-R-Env; CXCR4 tropic, AIDS Research and Reference Reagent website) into 293T producer cells. Both plasmids are based on the proviral clone PNL4-3. Both plasmids contain the firefly luciferase gene in the *nef* position; they are *env* negative due to a frameshift near its 5' end. PNL-Luc-R-Env- has a frameshift in *vpr* that prevents its production. The viral constructs were concentrated, diluted in PBS at $7-8 \times 10^7$ CFU/ml, reconstituted with a recombinant human CD4 [5 μ M, NIH AIDS Reagent Program, (Bethesda, MD)] and injected into animals as inflammatory stimuli. This generated neuroinflammatory responses followed by DG neuronal damage in the hippocampus as reported elsewhere (Bodner et al., 2003). To do this, 8weeks old CXCR4-EGFP/SDF-mRFP biotransgenic mice were anesthetized with intraperitoneal injection of avertin (33 mg/kg), placed on a stereotaxic device (Stoelting, IL) and injected with an Hamilton syringe containing 2 μ l solution of HXB2 virus (gift from Dr. Thomas Hope, Dept. of Virology, Northwestern University) into the DG of the hippocampus at the following coordinates: 1.94 mm posterior to Bregma, 1.5 mm lateral to the midline and 2 mm ventral from the pia. In controls, we used injection of saline solution of comparable volume (2 μ l). At the end of the experiments, mice were anesthetized, and brains removed and subjected to vibratome sectioning. Following sectioning of the brains, expression patterns of CXCR4-EGFP and SDF-1-mRFP were analyzed at different time points. For the TUNEL assay we used the ApopTag Red in Situ Apoptosis Detection Kit (Millipore).

Electrophysiology

Sagittal and coronal brain sections (300 μ m) containing the dorsal surface of the hippocampus and visualizing CMS cells were obtained from CXCR4-EGFP mice and whole-cell patch-clamp recordings were performed from EGFP-positive cells as previously described (Bhattacharyya et al., 2008).

Quantification and Statistical Analysis

To visualize the aCMS migratory stream, mice of various ages up to a year (6 mice/each age) were used and 108 (40 μ m) thick brain sections containing the hippocampus were prepared from each hippocampus, so 216 sections per animal, in which 36/animal were used for analysis. For chemotaxis assay, 3 experiments were performed, and each experiment, 5 membranes/group were used and statistical differences were assessed by student *t*-test at $P < 0.05$. To examine CMS pathway in CXCR4 KO mice compared controls, 8 weeks old mice (6mice) were used. To examine response of aCMS cells to HIV associated-inflammation and

injury in the DG, 8 weeks old mice and 12 experimental mice were used, 3 mice per time point (24 h, 1, 3 weeks post HIV).

RESULTS

1. A sustained population of radial astrocyte like stem cells outside the SGZ of the DG can be identified in the brain of adult CXCR4-EGFP mice

We evaluated the normal expression of CXCR4 using confocal analysis of brain sections from 8 week old CXCR4-EGFP transgenic (tg) mice. As expected, based on previous observations in the literature, we observed strong CXCR4-EGFP expression in the major adult NSC niches, the SVZ of the LV (Figs. 1A,B') and in the SGZ of the DG in the hippocampus (Figs. 1A,B''). In the DG, although CXCR4-EGFP expression is maintained by stem/progenitor cells in the SGZ throughout adulthood, no CXCR4-EGFP was observed in mature granule cells, indicating that CXCR4 expression is downregulated at these later stages of development (Tran et al., 2007). These results indicate that it may be possible to exploit the expression of CXCR4 to identify potential stem/progenitor cells in other areas of the adult brain. Indeed, our attention was drawn to a stream of EGFP expressing cells emanating from the LVs and apparently migrating towards the DG of the hippocampus (Figs. 1C,D). These cells appeared to originate in a discrete area of the medial wall of the LVs near the FDJ (Figs. 1C–E). The cells appeared to enter and navigate the fimbria (Figs. 1C–E), the meninges lining the fimbria (Figs. 1C,D,E') and the hippocampal fissure (Figs. 1C,D,E''), ultimately reaching the junction between the meninges and the tip of the granule cell layer of the lower blade of the DG (meningeal-DG junction, MDJ; Figs. 1C,D,E'''). Some of the cells accumulated at the MDJ and, in some instances, were found forward of the MDJ directly contacting the tip of the granule cell layer of the lower blade of the DG (Figs. 1C,D,E'''; Supporting Information Fig. S1E), forming an apparent cellular bridge between the LVs and the DG. This suggests that these cells may populate the DG in certain circumstances. As this migratory stream takes a caudal route in adult brain we named this novel migratory stream the aCMS and the cells expressing CXCR4-EGFP in this migratory stream aCMS-EGFP cells. This differs from the CMS described by a population of cortical interneurons in the developing mouse forebrain (Yozu et al., 2005). The aCMS and its different components are maintained throughout adulthood as they could be visualized at various ages (1 week up to 12 months), and from all sectional directions, in coronal sections showing the anterior and the posterior arms of the hippocampus, in sagittal sections along the mediolateral axis of the brain, as well as in coronal sections across the hippocampus (Supporting Information Fig. S2). Staining with an antibody against laminin that recognizes the basement membrane of the meninges further confirmed the close juxtaposition between the meninges and aCMS-EGFP cells (Supporting Information Fig. S1), suggesting that the meninges might specifically attract these cells. aCMS-EGFP cells displayed different morphologies as they moved through the different compartments of the aCMS. They displayed uni- or bipolar morphology typical of migratory cells (Figs. 1E,E'; Supporting Information Fig. S1D) as they exited the medial wall of the LV and entered the FDJ and the fimbria. On reaching the meninges bordering the fimbria and the ventral hippocampal fissure, some EGFP cells assumed a highly polarized radial like morphology resembling the radial glia found in the SGZ of the DG (Figs. 1E'–E'''; Supporting Information Fig. S1C–E;

Sild and Ruthazer, 2011). Specifically, two distinct types of radial glia that differed in morphology could be identified along the meninges. The first of these had its cell soma in contact with the meninges and a long radial process with multiple arborized endings that extended all the way to the pial parenchyma in the fimbria and the DG (Figs. 1E',E''; Supporting Information Fig. S1C). A second type had its cell body and arborized endings both in contact with the meninges (Figs. 1E''; Supporting Information Fig. S1D). Thus, this radial glia like cells spanned two anatomical layers; the meninges and the hippocampal parenchyma.

To determine the specific cell types expressing CXCR4-EGFP within the aCMS pathway, we performed immuno-staining and quantification studies with markers of mitotic and postmitotic cells (i.e., radial astrocyte NSCs, neural progenitors, and markers of NSC proliferation as well as markers for oligodendrocytes, astrocytes, neurons and immune cells). Using selected postmitotic markers (NG2, olig-2, IBA-1, CD45, NeuN), we found that these markers were not expressed by EGFP cells (Supporting Information Fig. S3). However, as for NSCs in the SVZ and the SGZ, we observed that most (more than 90%) EGFP cells within the fimbria and along the meninges expressed the radial glia markers BLBP (Figs. 2A–A''), and GFAP (Figs. 2B–B''), the neural progenitor marker nestin (Figs. 2C–C'') and the neuronal stem cell marker SOX-2 (Figs. 2D–D''). In these experiments, 36 brain slices/animal were analyzed and an average of (45 6 5) GFP cells/slice and (2270 6 145) GFP cells/mm³ in the fimbria were found. Using nestin-EGFP tg mice we confirmed the presence of nestin-EGFP expressing cells along the different compartments of the aCMS pathway (data not shown). Furthermore, immunostaining for the T-box brain protein-2, (Tbr-2), a specific marker for intermediate neuron progenitors, showed that the majority of CXCR4-EGFP cells (~80%) expressed Tbr-2 (Figs. 2E–E''). Thus, aCMS cells exhibit characteristics of radial glial like NSCs.

In the SVZ and the SGZ, NSCs self-renew and produce cell progeny through intermediate transit-amplifying progenitor cells, which lack self-renewal ability. To evaluate the proportion of proliferating cells within the aCMS population, we injected BrdU 2 hours before sacrificing the mice. Immunostaining for BrdU revealed that some CXCR4-EGFP cells (~25%) in the fimbria incorporated BrdU (Figs. 2F–F'''), and that this incorporation was not detected at 1, 2, and 3 weeks post BrdU labeling (data not shown), indicating that these cells are transient-amplifying progenitors. Furthermore, using BrdU Labeling together with SOX-2 and Tbr-2 staining, we found that a number of CXCR4-EGFP in the fimbria co-stained for BrdU and SOX-2 (Figs. 2G,H), and for Tbr-2 (Figs. 2I–I'''), indicating that a proportion of CXCR4-EGFP cells of the aCMS are transient-amplifying neuronal progenitors.

To confirm the migratory potential and origin of aCMSEGFP cells, and to demonstrate that these cells are normally present in wild type animals and not only in tg mice, we performed labeling studies in wild type and CXCR4-EGFP mice using intraventricular injection of a retrovirus expressing the red fluorescent protein mCherry. Consistent with the expression patterns of aCMS cells in CXCR4-EGFP mice, analysis of retrovirus injected brains revealed the presence of mCherry (red) labeled cells forming a continuous stream resembling CXCR4-EGFP cells along the aCMS pathway (Figs. 3A–D). 2 days post viral

injection, red labeled cells were found at the site of the injection in the VZ, indicating successful transduction of the cells by the mCherry retrovirus (Fig. 3A). 5 days later, mCherry positive cells with uni or bipolar morphology were noticeably distributed along the aCMS pathway (Figs. 3B–D), navigating the adjacent fimbria (Fig. 3C) and the meninges of the hippocampal fissures (Fig. 3D). At the meninges,

mCherry cells adopted a radial glia like morphology (Fig. 3D). However no red cells were observed within the DG at this time point (5days post retroviral injection). Similar to CXCR4-EGFP cells, red labeled cells within the fimbria also express GFAP (Fig. 3C'). Furthermore, when CXCR4-EGFP mice were injected with mCherry virus we observed mCherry in cells expressing EGFP that were distributed along the aCMS (Figs. 3E–H), indicating that mCherry is marking CXCR4-EGFP cells.

Overall these data identify the existence of a novel population of migratory cells that originated in a specific area of the VZ adjacent to the fimbria, at the FDJ, and migrate along the aCMS pathway that extends through the meninges, potentially reaching the DG and express markers shared by radial glia, and stem cells.

2. CXCR4-EGFP tg mice identify a previously unrecognized sustained neurogenic zone in the medial walls of the LVs

To characterize this population further, we sought to anatomically determine the precise source of these cells. We took advantage of CXCR4-EGFP mice and visualized the medial walls of the LVs from all sectional directions (Supporting Information Fig. S2). This allowed us to examine the distribution of aCMS-EGFP cells at different mediolateral levels throughout the hippocampus and to anatomically trace these cells to their origin. This appeared to be a region located beneath the hippocampus along the medial walls of the LVs between the dorsal surface of the hippocampus and the choroid plexus (Figs. 4A–A'', a–a''). We therefore named this region the SHZ. The SHZ could be easily visualized in whole mount preparations (Figs. 4B, B', B'', E–H). In these preparations we observed the presence of a 60-70um thick structure containing densely packed and highly fluorescent CXCR4-EGFP cells with almost no extracellular space, resembling cells in the SVZ (Figs. 4B–B'', Supporting Information Fig. S4). We also observed chains of single CXCR4-EGFP cells exiting the SHZ, which then appeared to penetrate the fimbria on their way toward the DG (Figs. 4C–C'', Supporting Information Fig. S4), and some of them expressed the intermediate neuronal progenitor marker Tbr-2 (Figs. D–D'), consistent with the results presented in Figures 1 and 2. To demonstrate that some of the EGFP-CXCR4 cells in this region were NSCs, we prepared whole-mount preparations and examined the coexpression of EGFP with several NSC markers, including nestin, and SOX-2, as well as proliferation markers (BrdU and Ki-67). We found that virtually all CXCR4-EGFP cells in this structure colocalized with nestin (Figs. 4E, E', E''), GFAP (Figs. 4F, F', F'', F''') and SOX-2 (Figs. 4G, G', G'', G'''). They also stained for BrdU following repetitive additions of BrdU over a period of 6 days (Figs. 4H, H', H'', H''', I, I''), indicating that they were slowly dividing. Consistent with this the majority of the cells also stained for Ki-67 (Figs. 4I', I''). A small proportion of BrdU positive but Ki-67-negative cells (Fig. 4I'') were also found, indicating that they had exited the cell cycle, most likely in preparation for migration and

differentiation. We also made whole cell patch clamp recordings from CXCR4-EGFP cells using SHZ whole mount preparations. Several studies have indicated the early expression of GABA-A receptors by neural progenitors. Indeed, GABA produced clear bicuculline reversible inward currents in these recordings (Fig. 4J). In addition, SDF-1, the chemokine ligand for CXCR4 receptors produced an inward current that was also antagonized by bicuculline, a property we have previously demonstrated in recordings from neural progenitors in the SGZ (Fig. 4J). Altogether these data demonstrate that EGFP cells in the SHZ have morphological, immuno-logical and functional characteristics of neural/stem cells consistent with the properties of the aCMS migratory cells described above (Figs. 1 and 2), indicating that this region is the source of the cells found along the described aCMS.

Having shown that CXCR4-EGFP cells in this region displayed the characteristics of NSCs, we reasoned that as with NSCs from the SVZ and the SGZ, the cells should form neurospheres (NSs) in cultures. We therefore examined the ability of the cells to self-renew using neurosphere assays under defined proliferative conditions. After 10-15 days in culture EGFP expressing cells dissected from SHZ whole mount preparations were also able to grow, expand and to form primary and secondary free floating NSs (Figs. 4K, K', K⁰⁰⁰⁰). Using antibodies against CXCR4 and nestin, we demonstrated that EGFP positive cells uniformly expressed CXCR4 receptors (Figs. 4K', K⁰⁰⁰⁰) and costained for nestin (Figs. 4K', K', K[>]), further suggesting their identity as neural progenitor cells. We then used Fura-2-based Ca²¹ imaging of whole NSs to examine whether functional CXCR4 receptors were truly expressed by these cells. As can be seen in (Fig. 4L), the cells in these NSs exhibited clear Ca²¹ responses upon addition of SDF-1, indicating expression of functional CXCR4 receptors. We also carried out Fura-2 imaging on single cells dissociated from NSs and found that most EGFP cells (>95%) responded to SDF-1, as well as to another chemokine MCP-1 and to ATP which activates purinergic receptors (positive control; Fig. 4L'). Similarly, single EGFP-CXCR4 cells prepared from fresh SHZ preparations also exhibited transient increases in [Ca⁺⁺]_i upon addition of SDF-1 (data not shown), confirming that functional CXCR4 receptors are expressed by these cells.

To further confirm the migration of SHZ cells along the aCMS we performed time lapse recording using SHZ whole mount preparations from CXCR4-EGFP mice as shown in (Figs. 4B, B'). Images were acquired within an imaging window of 250 × 250 mm every 15s during a 5min recording period using a Yokogawa CSU-W1-based spinning-disk confocal operated by the SlideBook 5.5 software. Consistent with the reported bipolar migratory morphology, fluorescence imaging revealed rapid migration of CXCR4-EGFP cells within the fimbria from the SHZ towards the DG (Supporting Information Fig. S5).

Altogether, these data identify a previously unrecognized zone (-the SHZ) with neurogenic potential localized beneath the dorsal surface of the hippocampus. The cells that derive from the SHZ display migratory morphology and migrate along the aCMS pathway towards the DG. They express NSC markers and exhibit uniform expression of functional CXCR4 receptors. In culture, SHZ cells formed NSs under the same conditions as SVZ and SGZ NSs, making the SHZ a third neurogenic zone in the adult mouse brain.

3. SDF-1 is constitutively expressed in the meninges in close juxtaposition to CXCR4 expressing cells in the SHZ niche and along the aCMS

If CXCR4-expressing cells along the aCMS pathway have the characteristics of NSCs, we hypothesized that, as in other tissues, their migration should be regulated by endogenous sources of SDF-1. We therefore examined the distribution of SDF-1 expression along the aCMS. Expression of SDF-1 was visualized with the aid of tg mice expressing an SDF-1-mRFP fusion protein (Tran et al., 2007; Bhattacharyya et al., 2008; Belmadani et al., 2009). As previously reported we observed that SDF-1-mRFP was highly expressed in the hippocampal formation (Figs. 5A,B) and in the hippocampus proper (Fig. 5C), and along the dorsal surface of the hippocampus in whole mounts (Fig. 5D). In order to examine sites of SDF-1 expression relative to aCMS-CXCR4 expressing cells, we crossed SDF-1-mRFP mice with CXCR4-EGFP mice and generated a new line of mice expressing both EGFP and mRFP. Using this line of mice, we observed that SDF-1-mRFP was expressed within the SHZ niche and along the aCMS pathway (Figs. 5A–D) in close proximity to migratory aCMS-CXCR4-EGFP cells (Figs. 5A'–D'), indicating that aCMS-CXCR4 cells may potentially respond to locally produced SDF-1. We observed strong expression of SDF-1 in the vasculature at the dorsal surface of the hippocampus where the SHZ niche is located (Figs. 5D',5-1D',5-2D'), within the fimbria where aCMS cells displayed migratory morphology (Figs. 5A'–C',5-1C'), as well as in the molecular layer of the DG (Figs. 5A–C). High levels of expression were also observed in the meninges where rows of aCMS cells were also found (Figs. 5A'–C',4-1A'). In the meninges SDF-1 exhibited two patterns of expression, as non-cell associated puncta, and within meningeal cells that resembled endothelial cells, consistent with previous suggestions that these cells normally produce SDF-1 (Reiss et al., 2002). In the parenchyma of the hippocampus, SDF-1 also exhibited a punctate pattern of expression (Figs. 51A,2A,1A',2A') which we have previously demonstrated corresponds with the nerve terminals of granule cells (Tran et al., 2007; Bhattacharyya et al., 2008), consistent with the idea that it is normally stored in neurotransmitter secretory vesicles.

4. Effects of SDF-1 on CXCR4-EGFP expressing cells of the SHZ niche

The phenotypes of CXCR4 KO mice indicate that SDF-1 influences the migration of progenitors during the development of many neural and non-neural tissues (Bagri et al., 2002; Lu et al., 2002; Stumm et al., 2003; Belmadani et al., 2005, 2009). Having established that CXCR4-EGFP-expressing cells in the SHZ expressed functional CXCR4 receptors and that SDF-1 was expressed along the aCMS, we addressed the possibility that SDF-1 might act as a migratory cue for these cells. We used a TMA (Belmadani et al., 2005) and prepared SHZ CXCR4-EGFP expressing cells from freshly isolated SHZ preparations to test their migratory response to SDF-1. After 45 minutes of incubation most plated SHZ cells were found below the membrane on the side of SDF-1 containing medium (Figs. 5F,J), whereas few cells were found in control medium (Figs. 5E,J) indicating that SHZ cells were attracted by SDF-1. The SDF-1 induced chemotactic effect on these CXCR4-EGFP cells was completely blocked by the CXCR4 antagonist AMD3100 (Figs. 5G,J). In addition, SHZ cells were also attracted towards the proinflammatory chemokine MCP-1 (Figs. 5H,J) which, as noted above, also produced [Ca²⁺]_i transients in isolated aCMS cells and has been shown to produce chemoattractant effects on NSCs (Belmadani et al., 2005, 2009; Tran et

al., 2005). In some experiments, dissociated SHZ cells were prepared from NSs with similar results (data not shown). Additionally, post hoc Fura-2 imaging of TMA migrated cells, showed an increase of $[Ca^{++}]_i$ in response to SDF-1, MCP-1 and ATP (Fig. 5I).

5. CMS cells increase in number and migrate in response to an inflammation or injury

Normally NSCs from the SVZ or SGZ of the adult brain respond to injury by proliferating and migrating towards sources of chemokines produced in association with tissue damage (Imitola et al., 2004; Cayre et al., 2009; Carbajal et al., 2010). To determine whether aCMS cells also migrate in response to inflammation or injury, we used a mouse model of HIV-1 infection as an inflammatory stimulus by injecting a variant of HIV-1 into the DG of the hippocampus of CXCR4-EGFP/SDF-1-mRFP mice and subsequently examined the expression patterns of CXCR4 and SDF-1. A single stereotaxic injection of a replication deficient X4-tropic virus into the hippocampus resulted in brain inflammation characterized by the infiltration of numerous white blood cells. At early time points post HIV-1 injection (24 h) we observed a dramatic change in the expression patterns of both SDF-1 and CXCR4. For example, 24 h following HIV-1 injection, there was a marked increase in the expression of CXCR4-EGFP in the ipsilateral DG (Figs. 6A,A'') compared to the contralateral DG (Figs. 6A,A'). This was accompanied by morphological changes to the stem cell pool of the DG. In particular, the radial glia cells and their processes in the SGZ appeared histologically altered and dystrophic (Fig. 6a'') compared to cells of the contralateral SGZ (Fig. 6a'). There were also significant changes in the expression pattern of SDF-1-mRFP in the ipsilateral hippocampus 24 hours post HIV-1 injection (Figs. 6B,B'') compared to the contralateral DG (Figs. 6B,B'). Flow cytometer analysis showed that the observed upregulated CXCR4-EGFP and SDF-1-mRFP was expressed by infiltrating monocytes in addition to microglia (Figs. 6C,C'). Importantly, by the second week following HIV-1 injection the expression of CXCR4-EGFP appeared to be attenuated and very few infiltrating cells could be observed (data not shown). In parallel with this, SDF-1-mRFP expression was also attenuated and mostly restricted to the vasculature in the parenchyma, and to cells of the meninges as described above in Figure 5. Strikingly the morphology of the ipsilateral DG 1 and 2 weeks following virus injection was markedly altered; the granular layer of the upper blade became much thinner, and the SGZ appeared to be absent owing to cell death as indicated by an increase in TUNEL positive cells (Figs. 6D',D''). In some instances, the entire upper blade of the DG was completely absent as could be seen using DAPI staining (Figs. 6D,D''). Most importantly following these changes, we observed an increased accumulation of migratory CXCR4-EGFP cells along the meninges and also in the vicinity of the upper blade and the hilus of the injured DG, within the first week (Fig. 6E) that appeared to continue at 2 weeks (Fig. 6E') and three weeks (Fig. 6E'') post HIV-1 injection. Immunostaining for GFAP, showed an upregulation of reactive astrocytes in the injured DG (Fig. 6F') that did not colocalize with the above described migratory CXCR4-EGFP cells (Figs. 6F,F'). Similarly, immunostaining for the immune cell marker CD45 showed the presence of CD45 cells in the ipsilateral DG, which was maintained at 2-3 weeks post HIV (Supporting Information Fig. S6). By 3 weeks, the streams of migratory CXCR4-EGFP cells continued towards the DG, and all experimental mice exhibited a morphologically normal DG including normal upper blades, granule cell layers and SGZ with its content of stem and progenitor cells (Fig. 6E''), suggesting that aCMS cells may be involved in an ongoing neuronal repair process.

To ascertain whether SHZ cells and their progeny in the aCMS were involved in the apparent repair process we used CXCR4-EGFP mice that were injected with mCherry retrovirus in the fimbria near the LV to label SHZ cells expressing EGFP as in Figure 4. These mice were then injected with HIV-1 and examined for the distribution of EGFP (green), mCherry labeled cells (red), and overlapping cells (yellow) at 2 weeks post HIV-1 injection. In control mice, SHZ cells (green, red, and yellow) were distributed along the aCMS pathway but no red or yellow cells were found within the DG (Figs. 7A,A', Supporting Information Fig. S7), as described above in Figure 4. However, in HIV-1 injected mice, many yellow cells were found along the different compartments of the aCMS such as in the LV (Figs. 7B,B'), in the fimbria (Figs. 7B,B''), and a large population of cells was found along the meninges (Figs. 7C,C') in the direction of the ipsilateral DG (Figs. 7D,D',D''). Some red and yellow cells were also found in the upper blade of the DG and in the molecular layer as well as in the hilus (Figs. 7D',D''). Consistent with this, immunostaining for GFAP (activated astrocytes), IBA-1 (activated microglia) and CD45 (monocytes) reveal that mCherry labeled cells have migrated away from the initial site of retroviral injection in destination of the DG after HIV injection (Figs. 7E,E',E''). Thus, in response to neuroinflammation and injury of the DG, CXCR4/SDF-1 signaling was dramatically altered followed by significant accumulation of aCMS cells in the DG. This may be part of a repair process resulting from directed migration of cells migrating from SHZ towards the injured DG and the hilus.

6. New born granule cells are produced following interruption of SDF-1/CXCR4 signaling

As discussed above, mCherry retrovirus labeling of SHZ cells revealed that aCMS cells were distributed along the meninges, with a tendency to accumulate at the meningeal-DG junction under normal conditions. The close juxtaposition of aCMS cells next to areas of SDF-1 expression in the meninges and at the meningeal-DG junction suggests a role for SDF-1/CXCR4 signaling in the normal location and positioning of aCMS cells. We therefore examined whether inactivation of CXCR4-SDF-1 signaling in aCMS cells would result in changes in their distribution. Animals in which CXCR4 receptors have been inactivated die in late gestation, just prior to birth (Nagasawa et al., 1996; Zou et al., 1998). We therefore prepared animals in which the CXCR4 receptor had been selectively deleted from nestin expressing cells and their progeny by crossing nestin-Cre with CXCR4^{fl/fl} mice (Tronche et al., 1999; Knoepfler et al., 2002; Michalczyk and Ziman, 2005). Deletion of CXCR4 from nestin expressing cells was confirmed by PCR using nestin expressing NSs. Additionally, Fura-2 based (Ca⁺⁺)_i assays performed on CXCR4 KO NSs showed a complete absence of Ca signals in response to SDF-1 stimulation (data not shown). The nestin-Cre mediated deletion of CXCR4 resulted in viable, fertile adult mice, with no obvious behavioral or motor phenotypes. Interestingly, the DG in these animals appeared normal (Supporting Information Fig. S8) in spite of the fact that it has been clearly demonstrated that interference with CXCR4 signaling in this cell population results in initial disruption of DG formation (Bagri et al., 2002; Lu et al., 2002). However, our observations are consistent with those of Li et al. (2009) who also demonstrated that the deletion of CXCR4 from NSCs resulted in a normal DG following an initial disruption. These authors demonstrated that the ability of the DG to eventually form properly was dependent on PTX sensitive signaling processes other than CXCR4 signaling.

To investigate the distribution pattern of aCMS cells in CXCR4 KO mice, we injected mCherry retrovirus into the LVs to label SHZ and examined the location and the positioning of SHZ mCherry labeled cells as in Figures 3 and 7. In control mice 5 days after viral injection we observed red cells with localization and morphology as described above, i.e. in the fimbria, along the meninges and at the meningeal-DG junction but no red cells were observed within the DG (Fig. 8A) as described in the above experiments (Figs. (3 and 7)A,A'). In CXCR4 KO animals this pattern was substantially altered (Fig. 8B). We now observed a substantial number (two to three fold increases) of red labeled cells not only along the aCMS pathway, but also within the DG (Fig. 8B). Red labeled cells were found in the hilus and within the extent of the granular cell layer of the DG (Figs. 8C, Supporting Information Fig. S9). Some of the cells identified within the DG maintained their nestin expression (Fig. 8D) and lost their GFAP (Supporting Information Fig. S9) but the majority exhibited simplified neuronal morphology with one or two short processes and stained for the granule cell marker Prox-1 (Fig. 8E). We further found that these cells didn't appear to stain for calbindin or for calretinin (Supporting Information Fig. S9). aCMS cells found in the hilus also stained for Prox-1 as well as for the neuronal nuclear marker NeuN (Fig. 8F), indicating that they were abnormally located granule cells.

In summary, aCMS cells become dispersed within the DG of CXCR4-KO mice suggesting that SDF-1/CXCR4 signaling normally helps to retain these cells at the meningeal-DG junction. The observation that some of the cells in the DG were abnormally localized under these conditions might suggest that disruption of CXCR4 signaling is one mechanism for the generation of ectopic granule cell localization in the brain in the context of diseases such as epilepsy and schizophrenia.

DISCUSSION

In the present series of experiments we identified the existence of a third neurogenic zone in the adult brain using tg mice that express EGFP under the control of the CXCR4 promoter. This region, designated the SHZ, is situated along the medial walls of the LVs between the dorsal surface of the hippocampus and the choroid plexus. In addition to its location in the VZ the SHZ exhibits morphological, immunological, and functional characteristics (Figs. 1–3) that are shared by the two natural endogenous neurogenic niches, the SVZ and SGZ. We also identified the presence of a population of migratory progenitor cells that appeared to originate from the SHZ. This population migrated caudally along the meninges over long distances in the direction of the DG. We named this migratory stream as the aCMS. In addition to CXCR4, aCMS cells also expressed the intermediate transient proliferation neuronal marker Tbr-2 and other markers expressed by NSCs (Fig. 2). On exiting the SHZ, these cells migrate away from the SHZ and enter the fimbria eventually arriving at the meninges near the ventral hippocampal fissure and became closely associated with other meningeal cells (Fig. 1 and Supporting Information Fig. S1). Once in the meninges some aCMS cells exhibited radial glial morphology and a tendency to accumulate in the junction between the meninges and the tip of the lower blade of the DG (Figs. 1C,D,E''' and Supporting Information Fig. S1E), and in some cases, some of the cells were found forward of the meningeal-DG junction directly in contact with the tip with the lower blade, making them indistinguishable from SGZ NSCs (Figs. 1C,E''' and Supporting Information Fig. S1E),

and therefore forming what appears to be a steady stream of migratory cells from the SHZ in the LVs and the SGZ in the DG consistent with our live imaging study (Supporting Information Fig. S5). The migration of these cells can be observed at all postnatal ages, at least from the first week of the postnatal life, during which the DG is still developing, and continued into adulthood (i.e., 12 months of age, the latest age analyzed in this study). This sustained migration of cells is very marked, and it is possible that it reflects a continued remnant of the embryonic sources of dentate stem cells. Recently, it has been shown that the SGZ receive progenitors from two spatially different embryonic regions controlled by distinct sources of Sonic Hedgehog, the first region being the DP during embryonic life (Altman and Bayer, 1990; Li et al., 2009; Hodge et al., 2012a, 2012b), and the second, being the ventral hippocampus near the amygdalo-hippocampal region, which, during late in gestation, generate progenitors that populate the SGZ to become the long lived NSCs (Li et al., 2013). The detailed migratory route of these cells and the point in development when these cells stop being produced are not currently known. The migration route of CXCR4-EGFP cells described in this study appeared to exhibit features that do resemble the presumed origin of dentate NSCs during embryonic life, and it is possible that this may reflect the continued production of these cells in the adult, perhaps derived embryonically from the same caudal source, but now residing in a restricted area of the medial walls of the LVs near the FDJ.

In the meninges, this migration route is likely to be influenced by a number of cellular and molecular factors derived from both the meninges and the cerebrospinal fluid (CSF) (Decimo et al., 2012). The interplay between these factors may ensure normal migration and positioning of aCMS cells. Different cell populations have been previously described in the meninges (Decimo et al., 2012). Recently, a population of cells with many of the features of NSCs was identified in the meninges of rat brain (Bifari et al., 2009; Decimo et al., 2011; Nakagomi et al., 2012). These cells could be expanded in vitro as NSs and differentiated into neurons in vitro and in vivo (Bifari et al., 2009). Importantly these cells could also be activated following spinal cord injury (Decimo et al., 2011). As in the rat, it has been demonstrated that a cell population in the leptomeninges of mice can exhibit stem/progenitor cell activity with neuronal differentiation potential in response to ischemia (Nakagomi et al., 2011) and stroke (Nakagomi et al., 2012). However, the origins of these cells are not known. We have now demonstrated the presence of a population of stem/progenitor cells (aCMS cells), that is different from those in the leptomeninges in the sense that these cells are not meningeal cells but have their origin at the ventricular neurogenic SHZ and are recruited to the meninges of the ventral hippocampal fissure.

To gain further insights into the mechanisms that regulate aCMS cell recruitment to the meninges we took advantage of bicolor SDF-mRFP/CXCR4-EGFP tg mice to illustrate that the chemokine SDF-1, the only natural ligand for CXCR4, was widely expressed along the migratory path of CMS cells, particularly along the meninges, suggesting that the meningeal expression of SDF-1 may play an important role in aCMS cell migration and positioning. One possibility is that, as in other circumstances, SDF-1 acts as a chemoattractant for aCMS-CXCR4 cells as demonstrated by our cell culture experiments (Fig. 5). During early cortical development, Cajal-Retzius (CR) are the first cells to utilize meningeal-derived SDF-1 to reach the marginal zone where they produce and secrete the protein reelin, an

important modulator critical for correct laminar positioning of neurons in the cerebral cortex (Borrell and Marin, 2006; Frotscher et al., 2009). Later in development, interneurons migrate from their birthplace in the ventral forebrain to the cortical plate under the influence of meningeal SDF-1 (Stumm et al., 2003; Li et al., 2008; Lopez-Bendito et al., 2008). In addition to CR cells and interneurons, there are other instances where SDF-1 is required for proper migration and positioning of neural precursors next to the meninges. For example, in both the developing cerebellum and DG, granule cell progenitors are positioned within a neurogenic zone by meningeally derived SDF-1 (Ma et al., 1998; Bagri et al., 2002; Lu et al., 2002; Li et al., 2009). In these instances SDF-1 was proposed to control cell migration and it was observed that removal of the meninges or inhibition of CXCR4/SDF-1 signaling halted cell migration and disrupted normal cell positioning.

We also used mutant mice lacking CXCR4 expression and injected with an retrovirus encoding mCherry to label SHZ cells and observed that, as early as 5 days post virus injection, while the majority of SHZ derived cells were distributed along the different compartments of the CMS pathway, a proportion of cells became dispersed within the granular cell layer and ectopically within the hilus of the DG. Interestingly, the mutant aCMS cells in the DG appeared to be neurons according to morphological and immunohistochemical staining for DG granule cell markers (Fig. 8, Supporting Information Fig. S9), indicating that, in addition to the SGZ, the SHZ can act as a source of new DG neurons under some circumstances. Although it is certainly clear that components of this pathway operate during the initial formation of the DG (Altman and Bayer, 1990; Li et al., 2009; Hodge et al., 2012b, 2013), this report is the first indication that this pathway still functions in the adult brain. Adult CMS cells originate in the ventricular SHZ, and migrate in a mediolateral direction following a meningeal route and ultimately accumulate at the meningeal junction or enter the DG, depending on the conditions. This suggests that the adult DG is a mosaic structure influenced by two neurogenic niches. One of these, the known SGZ, is nonventricular containing embryonically produced dentate granule neurons (DGCs). These are supplemented by adult DGCs originating in the ventricular SHZ.

Using a mouse model of HIV-1 mediated neuroinflammation and associated DG degeneration, in which normal SDF-1/CXCR4 signaling was disrupted; we observed migration of aCMS cells into the damaged DG associated with the recovery of this structure together with appearance of ectopically placed neurons (Fig. 6). This is consistent with previous studies showing the migration of NSCs from the SVZ or the SGZ can be altered in the presence of damage to the brain when these cells become involved in brain repair (Imitola et al., 2004; Belmadani et al., 2006; Cayre et al., 2009; Carbajal et al., 2010). It appears, therefore, that the SHZ can be called upon to provide the adult DG with newly formed granule neurons under conditions associated with brain pathology.

The observation that aCMS cells are also both normally and ectopically found in the DG following inhibition of CXCR4 signaling in neural progenitors suggests that meningeal SDF-1 functions to anchor these cells within the meninges, possibly at the meningeal-DG junction, via mechanisms of chemoattraction, and that dysregulated SDF-1/CXCR4 signaling occurring under conditions of neuroinflammation or injury may lead to the appearance ectopic neurons and aberrant hippocampal neuro-genesis. In the case of

neuroinflammation, for example, dramatic upregulation of SDF-1 levels may produce excessive stimulation of CXCR4 and desensitization of receptor signaling, resulting in downregulated CXCR4 signaling and consequent ectopic dispersion of newly born neurons in the DG as has been observed in a number of other situations (Wang and Baraban, 2007; Zhang et al., 2011; Niv et al., 2012). However, an accurate assessment of the effects of SDF-1 will also necessitate analysis of the concomitant role of CXCR7 receptors which can greatly influence the actions of the chemokine. CXCR7 may function as a SDF-1 scavenger to prevent overstimulation and desensitization of CXCR4 in the meninges, thereby indirectly regulating cell migration (Balabanian et al., 2005; Naumann et al., 2010; Sanchez-Alcaniz et al., 2011; Hoffmann et al., 2012; Wang et al., 2012). Indeed, using CXCR7-EGFP tg mice, we observed expression of CXCR7 along the meninges (Supporting Information Fig. S10). This being the case it may be that both inflammation and CXCR4 deletion can disrupt SDF-1/CXCR4 signaling resulting in increased migration and some ectopic placement of granule cell progenitors.

Ectopically placed DG granule cells have been observed to occur in the context of epilepsy or in schizophrenia (Wang and Baraban, 2007; Zhao and Overstreet-Wadiche, 2008; Fournier et al., 2010), although the mechanism underlying this phenomenon has not been elucidated. It seems possible that some of these ectopic cells may be derived from the progenitors described here following disruption of CXCR4 signaling. Indeed, as described by (Li et al., 2009) following disruption of CXCR4 signaling during development, the initial formation of the DG is compromised but then forms later on using PTX sensitive signals aside from CXCR4. Thus if CXCR4 signaling is compromised in the adult, it is likely that progenitors will not be retained properly in their meningeal locale and will migrate to ectopic positions in the DG using non CXCR4 dependent cues. It is interesting to note that abnormal CXCR4 mediated brain development may also be a significant factor in other disorders as well. For example, CXCR4 signaling regulates the migration of GABAergic interneurons into the developing cortex and interruption of this process may produce cortical abnormalities that give rise to some types of schizophrenia (Meechan et al., 2012).

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FIGURE 1.

Expression patterns of EGFP in the adult brain of CXCR4-EGFP tg mice, (See also Supporting Information Figs. S1 and S2): The panels on the left show the sectional profiles used to generate panels on the right showing representative sagittal (A, C) and coronal (B, B', B'', D) sections through the brain of a 2-months old CXCR4-EGFP mouse. EGFP expression is detected in the major neurogenic niches in adult brain; in the SVZ (A, B'), the OB (A, B) and in the SGZ of the DG (A, B''). In addition, streams of a sustained population of EGFP expressing cells with a migratory appearance are detected between the LV and the tip of the lower blade of the DG of the hippocampus as depicted in the area outlined by yellow dots, forming what is referred to as the aCMS. High magnification images of boxed areas in panel (D) are shown in (E-E'''), illustrating streams of migratory CXCR4-EGFP cells along the aCMS pathway; at the medial wall of the LV near the FDJ (E), in the fimbria (E, E') and along the meninges of the hippocampal fissure (E''), including the meningeal-DG junction (E'''), point of contact between the meninges and the lower blade of the DG. Note the continued and steady stream of CXCR4-EGFP cells between the FDJ and the SGZ of the DG and the changes of their morphology in the fimbria (E) and along the meninges in (E',

E"). Scale bars are 250 μm in panels A, C, and D; 200 μm in panels B, B', B'', and 20 μm in panels E, E', E'', and E'''. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 2.

CXCR4-EGFP cells along the aCMS pathway have migratory appearance and express stem/neural progenitor markers. A–H: Representative confocal images showing CXCR4-EGFP cells (green) with morphologies resembling migratory progenitors, in which a large proportion have un or bipolar morphologies, indicating their potential to migrate. A–A'': Example of CXCR4-EGFP cells with one or two long processes (green, A) stained for the radial glia marker BLBP (A', red). Merged images (A''). Immuno-staining for GFAP (B, B', B'') and nestin (C, C', C'') showed the coexpression of GFAP (red) and nestin (red) in GFP cells (green), in the fimbria (B, C), and along the meninges (B', C'), some with an apical radial glia process (arrows) with multiple endings at the meningeal-DG junction (B'', C''). Similarly, cells in the fimbria (D), along the meninges (D') and at the meningeal-DG junction also stained for the neuronal stem marker SOX-2 (red), including cells at the meningeal-DG junction (D''). Immunostaining for the neuronal progenitor marker Tbr-2, which showed its expression around the cell bodies and in the cell processes (E'), revealed that some of the cells in the SHZ and virtually all migratory cells within the fimbria (arrows, E, E'') stained for Tbr-2 (arrows, E', E'').

FIGURE 3.

To detect rapidly dividing cells, animals were injected with BrdU 2 h before euthanasia. Immunostaining for BrdU revealed that most of CXCR4-EGFP in the fimbria (arrows, F) incorporated BrdU (arrows, F'), indicating that these are rapidly dividing cells. Merged images (F''). X and Y projections of the cell in inset (F'') are shown in (F'''). Representative images of immunostaining for BrdU (red) and SOX-2 (blue) (High magnification), showing a number of transient amplifying neural progenitors (arrows) characterized by BrdU and SOX-2 expression in the SHZ (G, G', G'', G''') and the fimbria (H, H', H'', H'') of CXCR4-EGFP tg mice. Consistent with this, using BrdU (arrows, I', I'') with Tbr-2 (arrows, I', I''), we showed that these transient amplifying neural progenitors are fated to become neurons as characterized by BrdU and Tbr-2 expression in the fimbria (I, I', I'', I'''). Scale bars are 20 μ m in panels (As, Bs, C-C'', Ds, and Fs), 10 μ m in panels (C', Es, Gs, Hs, and Is). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 4.

mCherry retrovirus-mediated cell labeling of the LVs shows similar patterns as of CXCR4-EGFP expression. (Top panel) Scheme of mCherry retroviral labelling strategy that was used to label cells at the medial wall of the LV. mCherry retrovirus particles were injected into the LVs in adult brain of wild type and CXCR4-EGFP mice, and animals were euthanized 2 and 5 days later. The accuracy of the injection is indicated by red fluorescence at the LV in panel (A), which illustrates mCherry expression by cells of the LVs 2 days postviral injection. Five days later, streams of red mCherry- labelled cells with one or two processes were found along the aCMS pathway (B) as described for CXCR4-EGFP expression. High magnification images of boxed areas in panel (B) are shown in (C,D), showing mCherry-labeled cells (red) in the fimbria (Fi) (C) and the meninges (Me) of the hippocampal fissure (D). In the fimbria, red labeled cells display bipolar morphology (arrows, C). In the meninges, some cells displayed multiple processes morphology resembling radial glia (arrows, D). Immunostaining for GFAP shows that cells in the fimbria stained for GFAP (arrows, C'), and seemed to lack GFAP expression along the meninges

where they mainly adopt radial glia morphology (arrows, D'). E–H: Similarly, injection of a retrovirus-mCherry in the LVs of CXCR4-EGFP tg mice revealed that some mCherry-labeled cells along the aCMS migratory path (F), coexpressed GFP (E), indicating that retroviral-mCherry labeled cells are CXCR4-eGFP cells (yellow cells) (G). (G) counterstained with DAPI (blue) (H). (G' and H') are orthogonal projections showing the co-expression of mcherry and GFP in the same cell. DAPI counter-stain is shown in (blue, A, B). Scale bars are 250 μm in panel (A) and (B), and 20 μm in panels (C, D, C', D', E, F, G, G', H, and H'). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 5.

Expression of EGFP in a previously unrecognized region with characteristics of NSCs in the adult brain of CXCR4-EGFP tg mice. A-A'', a-a'': Experimental protocol used to dissect the dorsal surface of the hippocampus (DSH) from the brain in adult mice. The hippocampus was dissected as shown in (A-A''), and the DSH was displayed (a). (a', a'') DSH viewed at low power under an fluorescent binocular stereomicroscope equipped with a black and white CCD camera showing bright GFP fluorescence (a'), as shown by green dots in (a''). B-B'': Confocal reconstruction of representative whole mount preparations from areas highlighted in (a', a''), showing strong EGFP fluorescence in the SHZ. B: A face view of the DSH showing the location of the SHZ, also (B'). B'': Horizontal sections through the DSH showing strong EGFP expression (green) in the SHZ (left side of image) and the DG (right side of image) with some CXCR4-EGFP cells scattered between the SHZ toward the DG (B''), exhibiting long uni-or dual processes, suggestive of their capacity to migrate in direction to the DG (B''). Boxed areas in panels (C, C', and C'') show representative higher

magnification views of CXCR4-EGFP cells at the transitional area between the SHZ and the DG. C: Shows densely compacted cells in the SHZ with no apparent extracellular space. C' and C'': Shows streams of CXCR4-EGFP cells sometimes with long processes. D, D', and D'': Representative higher magnification image of these cells costained with the Tbr-2 antibody, indicating that these cells are neuronal progenitors. E–I: Whole mount preparations of the SHZ stained for EGFP antibody (green) showing CXCR4-EGFP cells also expressing a wide variety of stem cell markers (red). E–E'': Example of a whole mount SHZ preparation stained for the stem/progenitor marker nestin (red), showing that virtually all the EGFP cells (E) costained for nestin (E'); Merged image (E''). Whole mount SHZ preparations (F–F'', G–G'') and cross sections (F'', G'') showing that all SHZ cells (F, G), costained for GFAP (red, F) and the neuronal stem marker SOX-2 (red, G'); (F'', F'', and G'', G'') are merged images. To detect proliferating cells, animals were injected with BrdU, two times a day for 1 week, and euthanized 24 h later. Whole mount SHZ preparation (H–H'') and cross sections (H'') show that virtually all EGFP cells (H, I', green) incorporated BrdU (H', H'', H'', I, red), and also coexpressed the cell cycle marker Ki67 (I', I'', blue), indicating that most of the cells are slowly dividing. J: Electrophysiological characterization of SHZ cells showing similar functional characteristics to SGZ stem cells (Bhattacharyya et al., 2008). K: Neurospheres (NSs) obtained from cultures of SHZ whole mount preparations, indicating their self-renewal potential in cultures. Immunostaining of SHZ NSs for CXCR4 (K', K'') and nestin (K'', K'') demonstrates uniform expression of CXCR4 (blue) and nestin (red), further indicating the identity of SHZ cells as progenitor cells in cultures. L: Chemokines such as SDF-1 increased $[Ca^{2+}]_i$ in whole NSs, indicating the expression of functional CXCR4 receptors. (L') Example of three cells dissociated from SHZ NSs that show responses to SDF-1. Addition of another chemokine such MCP-1 also increased $[Ca^{2+}]_i$ as did the purinergic receptor agonist ATP. Scale bars are 2 mm in panels (A–A''), 500 μ m in panels a–a'', 250 μ m in panels (B–B''), 100 μ m in panels (EE'', F–F'', G–G'', H–H''), 100 μ m in panels (I–I''), and 20 μ m in panels (C–C''), and (D–D''), F'', G'', and H''). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 6. Expression patterns of mRFP in the brain of CXCR4-EGFP/SDF-1-mRFP tg mice: (A-D) Low power confocal images of representative sections from the brain of a 2-months old SDF-1-mRFP mouse. Representative sagittal (A) and coronal sections (1A, 2A, B) through the hemispheres; C) Coronal section through the hippocampus, and D) Face view of a whole mount SHZ preparations, showing wide punctate expression of SDF-1 in the parenchyma of the fimbria (Fi), along the meninges (Me) and in the hippocampal fissures (fis). Also there is strong expression of SDF-1 in meningeal cells of the hippocampal fissures (1A, 2A, 1A', 2A'), and in blood vessels (bv) (2A, 2A'). DAPI counterstain is shown in (blue, A, B, C, D, 1A, 2A). A'–D': Low power confocal images of a representative sections through the brain of a 2-months old CXCR4-EGFP/SDF-1-mRFP mouse, showing aCMS CXCR4-EGFP cells in close proximity of the cells expressing SDF-1, in particular along the meninges of the

fissure, indicating that they may potentially respond to SDF-1. A': Sagittal section, (1A', 2A', B') Coronal section through the hemispheres, (C) coronal section across the hippocampus, and (C') Face view of a whole mount SHZ preparation. The inserts (1C', 1D' and 2D') show high magnification views of boxed areas in panels (C') and (D'), showing the distribution of CXCR4-EGFP cells in relation to SDF-1 expression in the fimbria (1C'), along the meninges of the hippocampal fissure (1A'), and within the transitional area between the SHZ and the DG (arrows in 1D', 2D'). In (E-J) Chemotaxis assay showing strong chemoattractant responses induced by the addition of SDF-1 (100 nM) and its inhibition by the CXCR4 antagonist, AMD3100 (5 μ M) in cultured SHZ CXCR4-EGFP cells. Representative data from four experiments showing examples of cells that migrated in control membrane inserts (E), and in response to SDF-1 (F), SDF-1+AMD3100 (G), and also to another chemokine MCP-1 (500 nM; H). I: Post hoc Ca imaging experiment illustrating examples of three migrated SHZ-EGFP cells (three different colors) responding to SDF-1, MCP-1, and ATP, indicating sustained functional receptor expression. J: Migration index in different media conditions as defined as the quantification of the number of cells that had migrated into the bottom of the membrane. Values are means $\pm$ SEM of 6 fields/insert and four different experiments were used. Scale bars are 250 μ m in panels (A, A', B, B', C, C'), and (D, D'); 200 μ m in panels (1A, 2A, 1'A, 2A'); 20 μ m in panels (1C', 1D', 2D'). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 7.

HIV1-mediated inflammation of the hippocampus induced significant changes in CXCR4 and SDF-1 expression: (A) Experimental design that was used to infect the hippocampus by HIV1. CXCR4-EGFP/SDF-1-mRFP mice were injected with a solution of HXB2 virus into the DG of the hippocampus. Mice were euthanized at 24 h and 1, 2, and 3 weeks later. Histological analysis of hippocampal sections at 24 h post HIV1 revealed a massive infiltration of immune cells and an upregulated expression of CXCR4 (A, A', A'') and SDF-1 (B, B', B'') in the ipsilateral hippocampi (A, A'' and B, B'') compared to contralateral hippocampi (A, A', and B, B'). This was accompanied by morphological changes to the stem cell pool of the DG. In particular, the radial glia cells and their processes in the SGZ appeared dystrophic (a'') compared to cells of the contralateral SGZ (a'). Flow cytometry analysis of the infiltrating cells showed that most of the cells were CD45 positive (C', for CXCR4-EGFP and C'', for SDF-1-RFP) with the majority being macrophages as defined by CD11b high and F4/80 sorting (C' for CXCR4-EGFP, C'' for SDF-1-RFP). In (D-D'', E-E'') HIV1-mediated injury of the DG induced a directed migration of cells expressing CXCR4; (D-D'') TUNEL assays revealed frequent cell death in the upper blade of the ipsilateral DG by 1 and 2 weeks post HIV. D: DAPI staining (blue), (D') TUNEL staining (red), merged images in (D''). E-E'': Apparent directed migration of CXCR4-EGFP cells towards the upper blade of the DG. While TUNEL positive cells were diminishing but still present, a population of migratory CXCR4-EGFP cells (arrowheads) was found in direction of the

ipsilateral DG at 1 week (E), 2 weeks (E'), and 3 weeks (E'') post HIV. In (F), higher power view of image (E), showing a population of migratory cells in direction to the injured DG. In (F') the same image in (F) was costained with a GFAP antibody (red), showing that these migratory cells (arrows in F) are not reactive astrocytes (arrows, F'). Scale bars are 250 μm in panels (A, B), 200 μm in panels (A'-A'', B'-B'', D-D'', and E-E''), 100 μm in panels (F-F') and 50 μm in panels (a', a''). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

FIGURE 8.

mCherry retrovirus-mediated labelling of the SHZ reveals that the SHZ and its descendants in the aCMS migrate into the injured DG in response to HIV1: (Top panel) Experimental design used. mCherry retrovirus particles were injected into the LVs of the brain of CXCR4-EGFP tg mice. After 1 week, mice were injected with a solution of HXB2 virus into the DG, and were euthanized 3 weeks later. Brain sections were prepared and analyzed by confocal microscopy to look for migratory CXCR4-EGFP (green), and mCherry labeled cells (red). (A-A') Injection of a retrovirus-mCherry into the LVs of control mice marks SHZ cells in CXCR4-EGFP tg mice and most mCherry- labelled cells were found along the aCMS migratory path, including the MDJ, intermingled with CXCR4-EGFP cells, and some coexpressed GFP (yellow cells; A) stained with DAPI (blue; A'). 3 weeks post HIV1 injection, a subset of red and yellow cells, including green cells were found along the aCMS path, including the medial wall of the LV (B, B'), in the FDJ (B''), and along the meninges of the hippocampal fissure (C, C'). High magnification image of boxed areas in (A and B) are shown in (B', B'', C', C') illustrating yellow cells at the LV (B'), the fimbria (B'') and

along the meninges (C, C'). Importantly, some red and yellow cells were also found within the DG in the granule layer and in the hilus (D', D''). D: shows the migratory path taken by SHZ green cells at the MDJ towards the DG after mCherry injection for labelling followed by HIV1CD4 injection as stimulus. (E-E'') enlarged panels of boxed area in (D) showing representative image of GFAP (E), IBA-1(E'), and CD45 (E'') staining. These show that most mCherry positive cells have migrated away from the initial site of viral injection. Scale bars are 500 μm in panels (A, A', D); 250 μm in panels (B, E-E''), and 20 μm in panels (B', B'', C, C').[Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]