Ontogeny of CX3CR1-EGFP expressing cells unveil microglia as an integral component of the postnatal subventricular zone

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The full spectrum of cellular interactions within CNS neurogenic niches is still poorly understood. Only recently has the monocyte counterpart of the nervous system, the microglial cells, been described as an integral component of neurogenic niches. The present study sought to characterize the microglia population in the early postnatal subventricular zone (SVZ), the major site of postnatal neurogenesis, as well as in its anterior extension, the rostral migratory stream (RMS), a pathway for neuroblasts during their transit toward the olfactory bulb (OB) layers. Here we show that microglia within the SVZ/RMS pathway are not revealed by phenotypic markers that characterize microglia in other regions. Analysis of the transgenic mice strain that has one locus of the constitutively expressed fractalkine CX3CR1 receptor replaced by the gene encoding the enhanced green fluorescent protein (EGFP) circumvented the antigenic plasticity of the microglia, thus allowing us to depict microglia within the SVZ/RMS pathway during early development. Notably, microglia within the early SVZ/RMS are not proliferative and display a protracted development, retaining a more immature morphology than their counterparts outside germinal layers. Furthermore, microglia contact and phagocyte radial glia cells (RG) processes, thereby playing a role on the astroglial transformation that putative stem cells within the SVZ niche undergo during the first postnatal days.

Keywords: microglia, CX3CR1, subventricular zone, rostral migratory stream, neurogenesis

INTRODUCTION

Most often neurogenesis occurs in discrete regions known as germinal or germinative zones (Götz and Huttner, 2005; Franco and Müller, 2013). Interactions of specific cellular and molecular components of the neurogenic niche determine the progeny output (Jones and Wagers, 2008; Pathania et al., 2010; Lim and Alvarez-Buylla, 2014). In postnatal germinal zones, such as the adult telencephalic subventricular zone (SVZ) (Lim and Alvarez-Buylla, 2014) and subgranular layer of the hippocampus dentate gyrus (Seri et al., 2004), a common set of cells with distinct features are observed (Miller and Gauthier-Fisher, 2009), including quiescent multipotent neural stem cell with astrocytic characteristics, support cells, intermediary progenitors, immediate progeny, blood vessels and a specialized extracellular matrix (Tavaoie et al., 2008; Miller and Gauthier-Fisher, 2009). In the last few years it has been demonstrated that the monocyte counterpart of the nervous system, the microglial cell, is a full component of neurogenic niches (Mercier et al., 2002; Sierra et al., 2010; Olah et al., 2011; Cunningham et al., 2013). However, its importance, function, and interactions are yet to be fully uncovered.

Microglial cells constitute the main mesoderm-derived macrophage population of the central nervous system (CNS) (Prinz and Mildner, 2011) and are distinguished from other CNS cell types by their small cell soma, as well as by the expression of specific macrophage markers (Vilhardt, 2005). Monocytes precursors generated in the yolk sac invade the early embryonic nervous parenchyma as ameboid microglial cells (Chan et al., 2007; Ginhoux et al., 2010, 2013). As development progresses, microglia within the CNS parenchyma undergo differentiation, changing from ameboid morphology into ramified cells, rather deceitfully known as resting state (Nimmerjahn et al., 2005). Ramified microglia are typically distributed throughout the adult, healthy CNS (Imamoto and Leblond, 1978; Cuadros and Navascués, 1998; Dalmau et al., 2003; Hanisch and Kettenmann, 2007). In the course of an insult microglia revert to an ameboid morphology, which usually indicates their active state (Perry et al., 1993; Hanisch and Kettenmann, 2007). Moreover, microglia are involved in several events of brain development, such as phagocytosis, neuritio- and synaptogenesis, synaptic pruning, myelination, astrocyte proliferation and differentiation, and vasculogenesis (Giulian et al., 1988; Pow et al., 1989; Hamilton...
and Rome, 1994; Presta et al., 1995; Honda et al., 1999; Navascués et al., 2000; Streit, 2001; Rochefort et al., 2002; Marín-Teva et al., 2004; Shin et al., 2004; Checchin et al., 2006; Bessis et al., 2007; Nakanishi et al., 2007; Paolicelli et al., 2011; Kettenmann et al., 2013). Recently, microglia have also been shown to play an important role regulating neural progenitor physiology (Monje et al., 2003; Ziv et al., 2006; Sierra et al., 2010; Arnò et al., 2014; Su et al., 2014).

Here we investigate the ontogenesis, distribution and cellular interactions of microglia residing in the early postnatal SVZ, and its anterior extension, the rostral migratory stream (RMS). This region represents the major neurogenic niche in the mammalian brain that generates mostly interneurons destined for the olfactory bulb (OB) from birth to senescence (Altman, 1969; Luskin, 1993; Lois and Alvarez-Buylla, 1994). During the first two postnatal weeks a peak on proliferation is observed within the SVZ and its main progenitor cell, the radial glia (RG), undergo a process known as astrocytic transformation (Voigt, 1989; Misson et al., 1991; Freitas et al., 2012). During astrocytic transformation a set of RG transforms into astrocytes destined to populate the overlying mantle layers and/or into resident astrocytes of the SVZ/RMS pathway. Our results reveal that at this critical period microglia is already present in this germinal layer and greatly outnumber the microglia cells observed in the overlying cerebral cortex (CTX). Besides, SVZ/RMS microglia exhibit a more protracted differentiation rate compared to the regions outside this germinal zone. Importantly, during the first postnatal week SVZ/RMS microglia interact with RGs, the putative stem cells of this niche, possibly using RG processes as scaffold for its migration. Furthermore, SVZ/RMS microglia seem to engulf RGs processes, thus playing a key role in RG astrocytic transformation and possibly acting on progenitor regulation.

**MATERIALS AND METHODS**

**EXPERIMENTAL ANIMALS**

CX3CR1-encoding the green fluorescent protein (EGFP) mice on the C57BL/6J background were purchased from Jackson Labs (Strain Name B6.129P-CX3CR1tm1Litt/J, stock number 005582). Wild type Swiss mice, raised in our own colony, were also used. For both strains, mice at postnatal day (P) 0 up to P7 were used in our analysis. All experiments were performed in conformity with NIH (National Institute of Health, USA) guidelines for animal care and in accordance with protocols approved by both, the Animal Use Committees at the University of Rochester (UCAR-2011-021) and the Committee of Ethics on Animal Handling and Care at the Federal University of Rio de Janeiro (CEUA/DAHEICB 052; ICB/CCS—UFRJ).

**Tissue harvesting**

Heterozygous animals (CX3CR1+/-/EGFP+) and Swiss mice at P1 and P7 (n = 12 animals for each age, both strains) were deeply anesthetized by isoflurane inhalation (chamber atmosphere containing 4% isoflurane). Upon cessation of reflexes, mice were transcardially perfused with phosphate-buffered saline 0.1 M (PBS, pH 7.4, Sigma Aldrich) and paraformaldehyde 4% (PFA, Sigma Aldrich, in PBS 0.1 M pH 7.4). Brains were dissected and post fixed in PFA 4% for 3–6 h at room temperature (RT). Histological sections (50–100 μm) were obtained in vibratome (Vibratome Series 3000, Vibratome Co.) and kept in PBS containing azide 0.1% (Fisher Scientific) at 4°C for immunohistochemistry analysis (see Section Immunohistochemistry).

**BrdU administration**

Short pulses of the thymidine analog BrdU (5-bromo-2’-deoxyuridine; Sigma Aldrich) were performed in order to evaluate microglia proliferation along the SVZ niche. CX3CR1-EGFP mice (P1 and P7, n = 6 animals for each age) received a single pulse of BrdU into the intraperitoneal cavity (i.p. injection; 150 mg Kg−1) and were euthanized 1 h after BrdU administration. Proliferative cells were revealed by immunohistochemistry (described below), using a primary antibody that reacts with BrdU incorporated into single stranded DNA.

**IMMUNOHISTOCHEMISTRY**

Histological sections were blocked for 1 h at RT in a PBS containing 0.1% Triton-X (Sigma Aldrich) solution added with 5% normal donkey serum (NDS, Vector Labs) and incubation with specific antibodies against microglial/monocyte markers (Iba1; 1:500, Wako, CD68, F4/80 and CD11c; 1:100, AbD Serotec), neuroblasts (DCX; 1:1000, Millipore) and astroglial lineage cells (GFAP; 1:250, Sigma Aldrich) was performed overnight at 4°C. Proliferative cells were revealed by using an anti-BrdU antibody (1:100, AbD Serotec). To allow labeling of nuclear DNA, before blockage, sections were treated for 1 h with HCl 1M (RT) under agitation (Tang et al., 2007). Staining was revealed by 2-hour incubation period (RT) with appropriated secondary antibodies conjugated to Cy3 or Cy5 fluorophores (1:250, Jackson ImmunoResearch). DAPI (4′,6-Diamidino-2-phenylindole, 1:1000, Sigma Aldrich) was used for nuclear counterstaining and slides were mounted with ProLong Antifade (Life Technologies).

**FLUORO-GOLD TRACER INJECTIONS**

Pups (P0 or P1) were anesthetized by isoflurane inhalation (chamber atmosphere containing 4% isoflurane), and under visual guidance, 100–200 nl of Fluoro-Gold (FG; hydroxystilbamidine methanesulfonate in 2% in deionized water; Fluorochrome, Englewood, CO) were injected unilaterally in the pial surface (1–0.5 mm from midline and 0.5 mm anterior to Bregma) using a glass micropipette (80–100 mm tip diameter) coupled to a pressure injector (Nanoliter 2000, WPI, Sarasota, USA). Animals were analyzed 2 or 7 days after pial injections (n = 6; injection site included cortical supragranular layers; animals with deep injections reaching the cortical subgranular layers or the white matter were discarded from analysis).

**STATISTICAL ANALYSIS**

Histograms are expressed as mean ± standard error (SEM). Raw data, obtained in distinct experimental approaches used in the
RESULTS

ANALYSIS OF CX3CR1-EGFP+ CELLS DEPICTS MICROGLIA AS A CELLULAR COMPONENT OF THE EARLY POSTNATAL SVZ/RMS

Confocal microscopy analysis of brain sections obtained from newborn mice (P1) reveals that CX3CR1-EGFP+ cells accumulate at the ventricular layers, VZ/SVZ (Figure 1A). CX3CR1-EGFP+ cells are also distributed in the RMS core (Figure 1B), and within the OB layers (Figure 1C). In contrast, we observe very few CX3CR1-EGFP+ cells in the cortical parenchyma (Figure 1D). In common, CX3CR1-EGFP+ expressing cells in the SVZ, RMS, OB and CTX display immature/amoeboid morphology (Figures 1A1–D1), regardless of the significative difference on cell density between these regions [SVZ: 35 × 10^3 ± 4.3 × 10^3; RMS: 19.3 × 10^3 ± 1.3 × 10^3; OB: 20 × 10^3 ± 3 × 10^3; CTX: 4.4 × 10^3 ± 0.6 × 10^3; CX3CR1-EGFP+ cells/mm^3; mean ± SEM; p < 0.05 for SVZ in comparison to RMS and OB, and for RMS and OB in comparison to CTX, and p < 0.005 for SVZ in comparison to CTX; 1way ANOVA Bonferroni’s Multiple Comparison Test] (Figure 1E).

We next asked if the CX3CR1-EGFP+ cells observed within the SVZ/RMS niche, OB and in the cortical parenchyma correspond solely to microglial cells, as the fractalkine receptor is also expressed by monocytes, subsets of natural killers and dendritic cells (Jung et al., 2000). Since the dendritic cell antigen CD11c was detected in a transgenic mice strain in postnatal SVZ cells that were also immunoreactive for microglial markers (Bulloch et al., 2008), we analyzed by immunohistochemistry if CX3CR1-EGFP+ cells were co-labeled by CD11c. Notably, the majority of CX3CR1-EGFP expressing cells present in the SVZ/RMS, OB and cortical parenchyma correspond to microglia, as only a few cells restricted to the pial surface, are co-labeled by dendritic cell marker CD11c (Figures 2A,B,B1,B2).

Remarkably, analysis of brain sections obtained from CX3CR1-EGFP mice demonstrates that part of CX3CR1-EGFP+ cells are not co-labeled by CD68 (Figures 2C–E). Of the total of microglial cells observed within the SVZ, CX3CR1-EGFP+/CD68+ cells corresponded to 30.3% and CX3CR1-EGFP+/CD68− cells corresponded to 69.7% (Figure 2I). No CX3CR1-EGFP−/CD68+ cells were observed. Similarly, immunostaining for Iba1 revealed that 42.6% of the microglia in the SVZ are CX3CR1-EGFP+/Iba1+ and 57.4% CX3CR1-EGFP+/Iba1− (Figures 2F–I), indicating that the SVZ microglia are a heterogeneous population (Olah et al., 2011).

At later stages (P7), CX3CR1-EGFP expressing microglia present in the SVZ retains their immature morphology (Figures 3A,A1). Despite the dense population of ramified microglia outside its borders, CX3CR1-EGFP+ cells along
the RMS also display immature/migratory morphology (Figures 3B,B1), similar to the microglial cells distributed within the OB layers (Figures 3C,C1). In contrast, we observe ramified CX3CR1-EGFP+ microglia spanning all the cortical layers (Figures 3D,D1), and at this age, no significative differences on CX3CR1-EGFP+ cell number are observed when comparing all analyzed regions [SVZ: \(33 \times 10^3 \pm 3 \times 10^3\); RMS: \(26.9 \times 10^3 \pm 3.7 \times 10^3\); OB: \(30 \times 10^3 \pm 3.7 \times 10^3\);CTX: \(36 \times 10^3 \pm 2.6 \times 10^3\); CX3CR1-EGFP+ cells/mm\(^3\); mean \pm SEM; \(p > 0.05\)].

We next sought to determine the microglial interactions with the typical cell types observed within the SVZ niche, namely the astroglial stem cell lineage (RGs and stem cell-like astrocytes/type B cells) and neuroblasts. Since previous studies show that tracer injections at the pial surface labels exclusively RG within the SVZ (Freitas et al., 2012), we took advantage of the fact that at birth many RGs still maintain a long process touching the pial surface (Misson et al., 1991; Alves et al., 2002) to label this cell population. The neuroanatomical tracer Fluoro-Gold was injected in the pial surface of newborn mice (P0) and we followed labeled RGs up to the first postnatal week (P7). Immunohistochemistry analysis of brain sections obtained from injected animals reveals the transcellular labeling of microglia (F4/80+ cells) neighboring labeled RG (Figures 6B,B1,B2). This is suggestive of a very intimate contact of microglia with RG, although we could not distinguish if this transcellular labeling was due to whole engulfment of RG by microglia, or partial phagocytosis of RG processes. Immunolabeling of RGs and astrocytes with GFAP antibody reveal a close apposition of

**FIGURE 2 | Antigenic heterogeneity of CX3CR1-EGFP+ cells morphology in early postnatal SVZ.** (A) DAPI counterstaining unveils the cytoarchitecture of cortical parenchyma and ventricular region. Dendritic cells (CD11c+, red) are restricted to the pial surface and some of them co-express CX3CR1-EGFP (B,B1,B2). Immunolabeling of brain sections obtained from CX3CR1-EGFP mice with CD68 (C–E, red) and Iba1 (F–H, red) demonstrate that some of EGFP+ cells (green) are not co-labeled by these common used microglia markers (white arrowheads). (I) The percentage of CX3CR1-EGFP+/CD68+ cells is 30.3% of the total of microglia observed and of CX3CR1-EGFP+/Iba1+ cells is 42.6%. SVZ: subventricular zone. Scale bars: 100 \(\mu m\) (A) and 10 \(\mu m\) (B1,B2,C–H).

**MICROGliaAL CELLs RESIding IN the SVZ NICHe ARE NOT PROLIFERATIVE DURING EArLY NEONATAl STAGES**

Once inside the CNS, microglia precursors spread within the neural tissue, a process that includes cell proliferation and/or migration. In order to determine the spreading dynamics of microglial cells in the SVZ niche, we accessed the proliferation status of CX3CR1-EGFP+ cells in the SVZ, RMS and OB during the first postnatal week. After a short pulse of BrdU (1 h before euthanasia), scarce CX3CR1-EGFP+/BrdU+ cells are observed in the lateral (lv) and olfactory ventricles (Olfv) of P1 mice (Figures 5B,C, respectively, and Figure 5H). Within the OB layers, only CX3CR1-EGFP+/BrdU− are observed, indicating that the majority of microglial cells in the neonatal SVZ niche is quiescent (Figures 5D,H). At P7, we observe few CX3CR1-EGFP+/BrdU+ cells in the ventricular region (Figures 5E,I). Along the RMS, dividing microglia are detected in its borders, as well as some BrdU fragments are engulfed by microglia (Figures 5E,I). Remarkably, in the OB BrdU+ cells distributed along the distinct layers are sparsely contacted by CX3CR1-EGFP+ microglia and some CX3CR1-EGFP+/BrdU+ are observed (Figures 5G,I).

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FIGURE 3 | Characterization of CX3CR1-EGFP\(^+\) microglia morphology and density in neonatal mice. In P7 mice, microglia present in the SVZ retains immature morphology (A,A1), differing from EGFP\(^+\) cells present in the adjacent areas (WM and Str). In the RMS, CX3CR1-EGFP\(^+\) cells also display immature/migratory morphology (B,B1). Similar morphology is shown by CX3CR1-EGFP\(^+\) cells in the OB (C,C1). Within CTX layers, ramified CX3CR1-EGFP\(^+\) microglia are homogenously distributed (D,D1). (E) At this age, no significative differences on CX3CR1-EGFP\(^+\) cell number are observed in the analyzed regions [SVZ: 33 \(\times\) 10\(^3\) \(\pm\) 3 \(\times\) 10\(^3\); RMS: 26.9 \(\times\) 10\(^3\) \(\pm\) 3.7 \(\times\) 10\(^3\); OB: 30 \(\times\) 10\(^3\) \(\pm\) 3.7 \(\times\) 10\(^3\); CTX: 36 \(\times\) 10\(^3\) \(\pm\) 2.6 \(\times\) 10\(^3\); cells/mm\(^3\); mean \(\pm\) SEM; \(p > 0.05\), 1way ANOVA Bonferroni’s Multiple Comparison Test]. EPL: external plexiform layer; GCL: granular cell layer; lv: lateral ventricle; Mi: mitral layer; WM: white matter. Scale bars: 10 \(\mu\)m.

FIGURE 4 | Macrophage markers cannot depict the entire SVZ microglial population. (A–C) Similar to newborn mice, some of EGFP\(^+\) cells (green) are not co-labeled by CD68 (red) (indicated by arrowheads). CX3CR1-EGFP\(^+\)/CD68\(^+\) cells correspond to 40.2\% of the microglia in the SVZ (G). Immunostaining with Iba1 (D–F, red) reveals that CX3CR1-EGFP\(^+\)/Iba1\(^+\) cells (arrows) correspond to 20.8\%. and CX3CR1-EGFP\(^+\)/Iba1\(^-\) cells (arrowheads) represent 51.9\% of microglia (G). Notably, some cells are solely labeled by Iba1, corresponding to 27.3\% of the SVZ microglia (round arrowheads). lv: lateral ventricle. Scale bars: 10 \(\mu\)m.

Microglia in the SVZ/WM border, where GFAP\(^+\) cells accumulate during their putative astroglial transformation (Figure 6C2). Interestingly, along the SVZ/RMS of neonatal mice (P7) microglia are conspicuously distributed,
FIGURE 5 | Microglia residing in neonatal the SVZ, RMS and OB are quiescent cells. (A) Schematic representation of brain parasagittal section obtained from neonatal mice that received a single, short pulse of BrdU (i.p. injections of BrdU; 150 mg kg\(^{-1}\)). Sections containing the SVZ, RMS and OB were processed for BrdU immunostaining. (B) Dividing (BrdU\(^{+}\) cells, red) are observed in the ventricular layers of P1 mice, but majority of SVZ microglia do not incorporate BrdU and only few cells are EGFP\(^{+}\)/BrdU\(^{+}\), as depicted by optical sectioning. In the RMS of P1 mice, CX3CR1-EGFP\(^{+}\)/BrdU\(^{+}\) cells are restricted into the olfactory ventricle (C), while in the OB, we do not observe any CX3CR1-EGFP\(^{+}\)/BrdU\(^{+}\) cell at this stage (D). At P7, CX3CR1-EGFP\(^{+}\) cells intermingle BrdU\(^{+}\) cells, but few proliferative microglia are observed in the SVZ (E, arrows) and in the borders of RMS (F, arrows). Within OB layers, few CX3CR1-EGFP\(^{+}\) cells do not incorporate BrdU (G, arrows). Quantification is shown, in absolute numbers, in H and I. EPL: external plexiform layer; GCL: granular cell layer; GL: glomerular layer; Iv: lateral ventricle; Mi: mitral layer; OB: olfactory bulb; Olfv: olfactory ventricle; RMS: rostral migratory stream; SVZ: subventricular zone; WM: white matter; VZ: ventricular zone. Scale bars: 10 \(\mu\)m.

most often intermingling with the astrocyte compartment and outside of the chains of migratory neuroblasts (Figures 6E, F1, F2).

DISCUSSION

Here we demonstrate that microglia present within the early postnatal SVZ represents a copious population, which outnumber cortical microglia population during neonatal stages (Figure 1E). Our observations also show that SVZ microglia exhibit a remarkable antigenic plasticity (Figures 2, 4) and quiescence (Figure 5), confirming and extending the concept of microglia regional heterogeneity (Carson et al., 2007; Olah et al., 2011). Furthermore, our analysis reveal that microglia is intimately associated to the astroglial compartment within the SVZ, since dye transfer between RG cells and microglial cells were observed, possibly a result of phagocytosis, and a spatial overlap with GFAP positive cells (Figure 6). This interaction could represent a direct microglia control over late cortical progenitors of the outer SVZ (Franco et al., 2012) or progenitors for interneurons of the OB layers (Merkle et al., 2007; Ventura and Goldman, 2007). Alternatively, suggest that microglia is involved in the astrocitic transformation of a subset of RG cells in the early postnatal SVZ/RMS as suggested in earlier publications (Schmechel and Rakic, 1979; Voigt, 1989; Misson et al., 1991; Alves et al., 2002; Freitas et al., 2012).

For several years, the presence of microglia within the early postnatal SVZ/RMS was either neglected (Dalmau et al., 2003), or undetected (Peretto et al., 2005). Only recently has microglia within the early SVZ been investigated (Shigemoto-Mogami et al., 2014). The previous underestimation of microglia within germinative layers, and specifically in the postnatal SVZ, may be due to the great phenotypic plasticity of microglia cells (Saijo and Glass, 2011), making detection by usual phenotypic markers unreliable. We have circumvented this limitation by using a transgenic animal in which the reporter gene encoding the green fluorescent protein (EGFP) was introduced in the locus of the constitutively expressed fractalkine CX3CR1 receptor (Jung et al., 2000), yielding a stable marker for this population. This results in a golgi-like cell labeling with EGFP, throughout the developing brain parenchyma, displaying several characteristics of microglia. CX3CR1-EGFP\(^{+}\) microglia in the SVZ were neither immunoreactive for neural nor blood vessel markers (data not shown), and also unlabeled by a dendritic cell marker CD11c. Interestingly and rather unexpected, common used
FIGURE 6 | Microglia cellular interactions within the SVZ niche.
(A) Schematic representation of brain parasagittal section obtained from neonatal Swiss mice injected with FG (2%) in the pial surface. (B) Histological sections of injected mice and immunolabeled for F4/80 antigen reveal the overlapping of FG⁺ cell soma (magenta) and F4/80⁺ cells (green) in the SVZ and in the WM. RGs present in both the SVZ and overlaying WM are depicted in higher magnification in (B1) and (B2), respectively. Optical sectioning shows that FG⁺ cells are engulfed by F4/80⁺ microglia. (C) Microglia (F4/80⁺ cells, green) appose to RG processes (GFAP, magenta) that span the cortical parenchyma. (C1) Microglia displaying migratory morphology appose to RG processes spanning the WM (arrowheads). (C2) Within the SVZ, microglia displaying immature morphology are closely associated to GFAP⁺ astroglial cells (round arrowheads) and F4/80⁺ cells (green). (D) In transgenic mice, CX3CR1-EGFP⁺ cells (green) also associate to RG processes (GFAP, cyan), displaying migratory morphology, thus using astroglial processes as scaffold to invade the cortical parenchyma. (E) Orthogonal view of CX3CR1-EGFP⁺ cells along GFAP⁺ processes (arrowheads). (F) SVZ and RMS delimit the cell dense region counterstained with DAPI. Within the SVZ, CX3CR1-EGFP⁺ cells intermingle GFAP⁺ cells (F1), whereas in the RMS, microglia cells interleave neuroblasts chains (F2, DCX⁺ cells, cyan).

markers for microglia only partially co-localize with CX3CR1-EGFP⁺ cells. Since most of phenotypic markers used to reveal macrophages are membrane molecules related to cell-cell or cell-extracellular milieu interactions (Ling et al., 1991; Milligan et al., 1991; Chen et al., 2002; Gomez Perdiguerro et al., 2013), this variability of antigen expression may reflect the influence of discrete signals present within this neurogenic niche, which may instruct and control identity and specialization of microglia.

The most striking morphological feature observed for microglia within the neonatal SVZ is their characteristic activated profile (Figures 1, 3A), exhibiting ameboid morphology with few thick and short branches, also typical of immature microglia (Perry et al., 1993; Hanisch and Kettenmann, 2007). At P7, when cortical microglia already display a ramified “resting” morphology (Lima et al., 2001; Dalmau et al., 2003), SVZ microglia still retains the immature/activated profile. This may be a common feature for microglia resident of germinative layers, since the same reactive profile has been described for the embryonic cerebral cortical VZ/SVZ (Cunningham et al., 2013) and adult SVZ (Goings et al., 2006). Another outstanding difference of SVZ microglia is their relative quiescence (Figure 5), in contrast to actively proliferating microglia distributed throughout the cortical parenchyma during the first postnatal week (Mallat et al., 1997; Alliot et al., 1999; Dalmau et al., 2003). These regional differences could result from signals emanating from a progenitor enriched environment that has been shown to instruct resident microglia (Mosher et al., 2012; Linnartz and Neumann, 2013). It remains to be determined if this microglia behavior is dynamically controlled or represents an irreversible phenotype. It is interesting to note that microglia harvested from the adult SVZ, behave differently in culture, even after many in vitro passages (Walton et al., 2006), suggesting some stable and environment independent features for this microglial population.

A straightforward mechanism for any putative function for microglia over SVZ/RMS progenitors could lie on their intrinsic phagocytic activity. Phagocytosis of neural progenitors has been
shown to occur in the subgranular layer of the dentate gyrus (Sierra et al., 2010) and in the embryonic telencephalic ventricular zone (Cunningham et al., 2013). To test this hypothesis we have retrogradely labeled RG cells present in the SVZ by injecting the fluorescent tracer Fluoro-Gold at the pial surface. Previous results have shown that 2 days after pial injection of anatomical tracers only RG are labeled within the SVZ (Freitas et al., 2012). Interestingly, at 7 days post injection, we find the labeling of microglia. This can be explained as reminiscent of transcellular transfer of dyes to microglia by phagocytosis of retrogradely labeled cells, as observed in other systems (Thanos et al., 2000). Together this data suggests that microglia is actively phagocytizing RGs. However, alternatively this transcellular labeling may be due to gap junctional communication (Freitas et al., 2012) or partial phagocytosis of RG processes in a manner analogous to microglia stripping of neuronal synapses and processes described previously (Kettenmann et al., 2013). Although further investigation may be necessary to distinguish between these possibilities, the transcellular dye transfer and overlap of distribution with the astroglial compartment, as shown by double labeling with GFAP (Figure 6C2), indicates a consistent interaction between microglia and RGs. Nevertheless, we cannot rule out the hypothesis that microglia may also be phagocytizing neuroblasts en route to the OB layers, as shown to occur at the hippocampus dentate gyrus (Sierra et al., 2010).

Given their rapid response to diffusible signals and cell-cell interactions, microglia may represent a pivotal player to integrate short and long-range environmental cues within the germinall layers (Su et al., 2014). It has been documented that microglia can respond to neurotransmitters (Fontainhas et al., 2011); trophic factors (Ryu et al., 2012); peripheral cytokines and chemokines (Bouvsky et al., 2006); humoral signaling from disease (Li and Graeber, 2012; Tsuda et al., 2013; Yu and Ye, 2014; Hu et al., 2015), membrane glycoproteins (Linnartz and Neumann, 2013) and progenitor secreted proteins (Mosher et al., 2012). On the executive side, microglia could exert their influence not only by its phagocytic activity, engulfing whole cells, processes, or stripping membranes (Kettenmann et al., 2013), but through the release of cytokines and trophic factors and (Nakajima et al., 2007; Cacci et al., 2008; Liao et al., 2008; Ueno et al., 2013). It is still unclear what specific roles microglia play over the generation (Shigemoto-Mogami et al., 2014), migration (Aarum et al., 2003) and addition of new neurons to the OB (Lazarini et al., 2012) in and response to insult (Goings et al., 2006). However, given the possible action of the selective phagocytosis of precursors and progeny (Sierra et al., 2010; Cunningham et al., 2013) microglia activity could contribute to the mismatch observed between the very restricted generative potential of SVZ neural progenitors in situ (Luskin, 1993; Lim and Alvarez-Buylla, 2014) and its wider capabilities when challenged in vivo or in vitro (Sequerra et al., 2010, 2013).

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