Loss of Cxcr5 alters neuroblast proliferation and migration in the aged brain

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Abstract
Neurogenesis, the production of new neurons from neural stem cells, dramatically decreases during aging concomitantly with increased inflammation both systemically and in the brain. However, the precise role of inflammation and whether local or systemic factors drive the neurogenic decline during aging is poorly understood. Here, we identify CXCR5/CXCL13 signaling as a novel regulator of neurogenesis in the aged brain. The chemokine Cxcl13 was found to be upregulated in the brain during aging. Loss of its receptor, Cxcr5, led to increased proliferation and decreased numbers of neuroblasts in the aged subventricular zone (SVZ), together with accumulation of neuroblasts in the rostral migratory stream and olfactory bulb (OB), without increasing the amount of new mature neurons in the OB. The effect on proliferation and migration was specific to neuroblasts and likely mediated through increased levels of systemic IL-6 and local Cxcl12 expression in the SVZ. Our study raises the possibility of a new mechanism by which interplay between systemic and local alterations in inflammation regulates neurogenesis during aging.

KEYWORDS
aging, Cxcl13, Cxcr5, neuroblast, neurogenesis

1 | INTRODUCTION

Neurogenesis, the formation of new neurons from neural stem cells (NSCs), persists throughout life in at least two adult neurogenic niches, the subgranular zone of the hippocampal dentate gyrus (DG) and the subventricular zone (SVZ) lining lateral ventricles. NSCs in the adult rodent SVZ produce transient amplifying progenitors (TAPs), which give rise to neuroblasts that migrate from the SVZ via the rostral migratory stream (RMS) to the olfactory bulb (OB) where they differentiate into interneurons.1,2

In aged mice, the number and proliferation of neural stem and progenitor cells (NSPCs) as well as neuroblasts is drastically decreased in the SVZ,3 neuroblast density is lower in the aged RMS, and the amount of newly formed cells in the OB decline.4,5

During aging, inflammation increases both systemically and in the brain. The blood brain barrier (BBB) becomes disrupted with age, which enables infiltration of circulating cytokines.6 Microglia, the brain resident immune regulating cells, become both more poised for activation and more activated.7 Importantly, inflammation and microglia are critically involved in neurogenesis and regulate NSPC proliferation and differentiation.8,9
However, how inflammation affects neurogenesis during aging and whether local or systemic inflammatory mediators are involved in the age-related neurogenic decline is poorly understood.

B and T cells in blood as well as cerebrospinal fluid (CSF) express the C-X-C chemokine receptor 5 (Cxcr5) and are attracted by its only known ligand CXCL13. In the brain, microglia and neurons can express Cxcl13 and levels of CXCL13 in CSF increase in some neurological disorders such as multiple sclerosis. Interestingly, Cxcr5 has also been suggested to regulate neurogenesis in adult zebrafish and in the murine DG.

Here, we show that loss of Cxcr5 leads to increased proliferation and decreased number of neuroblasts in the aged SVZ together with accumulation of neuroblasts in the RMS and OB. The effect on proliferation and migration was specific to neuroblasts, and we suggest an underlying mechanism mediated by increased levels of systemic IL-6 and Cxcl12 expression in the SVZ.

2 | MATERIALS AND METHODS

2.1 | Animals

All experimental procedures were approved by Malmö-Lund Ethical Committee for the use of laboratory animals and were conducted in accordance with European Union directive on the subject of animal rights. Male and female, equally distributed between groups, adult (3-4 months) and aged (18-24 months) Cxcr5−/− mice (Jackson Laboratory, http://jaxmice.jax.org/strain/006659.html) and C57bl/6 J (Jackson Laboratory), were bred and housed in the animal facility connected to Lund University Biomedical Center at 22°C, 40% to 60% humidity, and 12 hours light/dark cycle with ad libitum access to food and water.

2.2 | EdU and BrdU administration

Adult mice were injected with BrdU (20 mg/mL in phosphate buffered saline (PBS), 50 mg/kg) i.p. twice daily for 2 weeks. Four weeks later, the same mice were injected with EdU (10 mg/mL in PBS, 50 mg/kg) i.p. four times with 2 hours interval and sacrificed 2 hours after last injection to assess survival and proliferation, respectively.

One group of aged mice were injected with BrdU (20 mg/mL in PBS, 50 mg/kg) i.p. twice daily for 2 weeks, and sacrificed 4 weeks after the last injection to assess survival. Another group was injected four times with 2 hours interval and sacrificed 2 hours after last injection to assess proliferation.

2.3 | Immunohistochemistry

Animals were given an overdose of pentobarbital (Apotek produktion & laboratorium, APL), transcardially perfused with saline followed by ice cold 4% paraformaldehyde (PFA), postfixed over night, and cryoprotected in 20% sucrose in potassium phosphate buffered saline (KPBS) (2.745 g/L KH₂PO₄·H₂O, 16.03 g/L K₂HPO₄·2H₂O, 50.4 g/L NaCl). Brains were then cut into 30 μm coronal or sagittal sections using a microtome (Leica), and stored at −20°C in antifreeze solution (30 vol%/vol% Ethylene Glycol, 30 vol%/vol% Glycerol in KPBS). OBs were cut in 20 μm sagittal sections, directly mounted onto glass slides using a cryostat (Leica), and kept at −80°C.

Free-floating or OB sections on glass slides were washed and blocked for 60 minutes in potassium phosphate buffered saline with Triton (KPBS) (0.25% Triton X-100 [Sigma] in KPBS) containing 5% normal donkey serum (NDS) and/or 5% normal horse serum (NHS). Washed, and incubated with primary antibodies over night at 4°C in blocking solution. Washed again, and incubated with secondary antibodies (Jackson ImmunoResearch, Cy3 or Thermo Fisher Scientific, Alexa Fluor, 1:200) in blocking solution. Washed again, and incubated with biotinylated goat antimouse (Jackson ImmunoResearch, Cy3 or Thermo Fisher Scientific, Alexa Fluor, 1:200) in blocking solution. Washed again, and incubated with primary antibody.

For BrdU staining, after blocking, sections were treated with 2 M HCl at RT for 60 minutes. Sections were then washed and incubated with primary antibody.

For EdU stainings, after blocking, sections were treated with Click-iT (Thermo Fisher Scientific) reaction cocktail at RT for 30 minutes following manufacturer’s guidelines. Sections were then washed and processed as described above.

For Terminal deoxynucleotidyl transferase dUTP nick endlabeling (TUNEL) staining, after blocking, sections were treated with Click-iT Plus TUNEL (Thermo Fisher Scientific) reaction cocktail following manufacturer’s guidelines. Sections were then washed and processed as described above.

For BrdU staining, after blocking, sections were treated with 2 M HCl at RT for 60 minutes. Sections were then washed and incubated with primary antibody.

For BrdU/DCX and BrdU/NeuN stainings, after blocking, sections were incubated with DCX or NeuN primary antibodies over night at 4°C in blocking solution. Washed, and incubated with biotinylated secondary antibody (Thermo Fisher Scientific, 1:200) in blocking solution at RT for 2 hours, followed by another washing step and incubation at RT for 1.5 hours with fluorophore-conjugated streptavidin. Sections were then washed, fixed for 20 minutes in 4% PFA, washed

Significance statement

The results of this study show that inflammatory mediators can, without direct action on neural stem and progenitor cells, have profound and specific effects on neurogenesis. Loss of Cxcr5 influenced systemic and local cytokine expression, which was accompanied by changes in neuroblast proliferation and migration in the aged brain. This implies that naturally occurring alterations in systemic and local inflammation contribute to neurogenic decline during aging and that therapeutic interventions targeting cytokine signaling in inflammation could affect neurogenesis in the aged brain.
and treated with 2 M HCl at RT for 60 minutes followed by washing and incubation with BrdU primary antibody over night at 4 °C in blocking solution. Sections were then processed as described above.

For BrdU/SOX2 staining, sections were blocked for 60 minutes in 0.25% TKPBS containing 3% NDS, 3% NHS, and 0.2% bovine serum albumin. Sections were then washed and incubated with SOX2 primary antibody over night at 4 °C in KPBS, and further processed as for BrdU/DCX staining.

For BrdU/Mash1 (BD Pharmingen) staining, sections were quenched with 3% H2O2 and 10% MeOH in KPBS for 20 minutes at RT followed by washing and treatment with 2 M HCl in RT for 60 minutes. Sections were then washed, and blocked with 5% NDS and 5% NHS for 60 minutes at RT, followed by blocking with Fab fragments (Jackson ImmunoResearch, 1:40) in KPBS for 30 minutes at RT. After additional washing, sections were incubated with 15% streptavidin (Thermo Fisher Scientific, sp2002) in blocking solution for 30 minutes at RT. Next, sections were washed and incubated with 15% biotin in blocking solution for 30 minutes at RT, followed by washing and primary antibody incubation with Mash1 and BrdU over night at 4 °C in blocking solution. After washing, sections were incubated with Biotinylated secondary antibody in KPBS for 2 hours at RT, followed by treatment with the avidin-biotin complex (Thermo Fisher Scientific) (4 μL A, 4 μL B, 500 μL KPBS) for 1 hour at RT. Next Biotin-xx Tyramid (Thermo Fisher Scientific, 1:100) in amp buffer was applied for 6 minutes at RT, and incubated with streptavidin together with secondary antibodies for 2 hours at RT. Sections were then processed as described above.

2.4 Imaging and quantification

To show colocalization and acquire high magnification images, a Zeiss LSM 780 confocal microscope with a ×63 objective was used together with Zen software, for overview images of sagittal sections an Olympus VS120-26-096 Virtual Slide Microscope with a ×20 objective was used together with Olympus VS-ASW 2.9 software. All other analysis and quantifications were performed under a BX61 epifluorescence microscope (Olympus), and by a blinded observer.

Quantifications were performed in the lateral wall of the SVZ, defined as the cell dense area lining the lateral wall, along the whole ventricle starting 40 μm from the dorsolateral corner until 40 μm past the ventral corner as illustrated in Figure S1. The SVZ in both hemispheres was quantified in four sections for each animal and presented as mean total number of positive cells per section and hemisphere, where the total amount of cells was assessed for BrdU, SOX2, Mash1, DCX, IBA1, and ED1. When examining microglia, cells were divided in different groups depending on morphology as previously described,14 corresponding to their activation state, from ramified-, through intermediate-, to amoeboid- and finally round morphology, and presented relative to the total amount of IBA1+ cells.

Quantification in the RMS was carried out in sagittal section on three different levels (1.08, 0.96, and 0.84 lateral to Bregma). The total number of positive cells for either BrdU and DCX or TUNEL was quantified along the whole RMS and presented as mean per section.

For OB analysis, four representative sections were chosen and total amount of BrdU+ and double positive BrdU+/NeuN+ cells were quantified for the granule cell layer (GCL) and internal plexiform layers (IPLs), and presented either as ratio or density.

2.5 Dissection

Brains were collected as described.15 Briefly, animals were sacrificed by cervical dislocation and brains collected in ice-cold L-15 (Invitrogen) medium. For RNA isolation, whole brains were collected or sectioned coronally into 1 mm thick slices before SVZs were dissected. Tissue was then stored at −80 °C in RLT lysis buffer (Qiagen) supplemented with 1% 2-mercaptoethanol (Sigma). For cell culture, SVZ was subdivided from 4 Cxcr5+/− and control mice, pooled in ice-cold L-15 medium, respectively, and dissociated with the adult brain dissociation kit, mouse and rat (Miltenyi Biotec, 130-107-677) following manufacturer’s guidelines. Cells were then purified through a two-step gradient as previously described15 and used for neurosphere assays.

2.6 Cell culture and neurosphere assay

SVZ derived cells were pooled from 4 Cxcr5+/− and wild type (WT) mice, respectively, and maintained at clonal density (10 000 cells/mL) in in humidified incubators set to 5% CO2 and 37 °C. NSPCs from SVZ were expanded, as described15 in Dulbecco’s Modified Eagle Medium (DMEM)/F12 + L-glutamine/Glutamax (Gibco) supplemented with 2% B27 (Gibco), 1% penicillin streptomycin (Sigma), 10 ng/mL epidermal growth factor (EGF) and 20 ng/mL basic fibroblast growth factor (bFGF). Fresh media (25% of initial volume) with growth factors was added every second day. Number of primary spheres were quantified and measured by diameter using CellSense imaging software.

2.7 Cytometric bead array

Peripheral blood was collected from the neck, following decapitation, into heparin-coated tubes (Sarstedt), kept on ice and centrifuged at 2500 rpm for 15 minutes to obtain plasma. Samples were stored at −80 °C, and later analyzed using the Cytometric Bead Array, Mouse inflammation kit (BD biosciences) for cytokines IL-6, IL-10, MCP-1, IFN-γ, TNF-α, and IL-12p70 following the manufacturer’s instruction. Briefly, plasma was incubated with beads and antibodies before analyzed with flow cytometry (ARIA III). Data are presented as concentrations in pg/mL blood.

2.8 RNA isolation and RT-qPCR

Total RNA was isolated from whole brain and SVZ tissue or sorted Dcx-GFP+ neuroblasts using the RNeasy mini kit (Qiagen) according
to manufacturer’s instructions. Briefly, samples frozen in RLT lysis buffer were brought to RT, homogenized by pipetting, and transferred to QIA-shredders. On-column DNase digestion (Qiagen) was performed during RNA purification, and the final elution of RNA was carried out twice with 30 μL H2O to a final volume of 60 μL. Concentrations were measured using a Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific).

To obtain cDNA, 300 ng RNA was used together with the qScript cDNA Synthesis Kit (Quanta) in a thermal cycler PTC-200 (Biorad) following manufacturer’s guidelines. 96-well plates (Sarstedt) were prepared with 2x TaqMan Master Mix (life technologies), 20x TaqMan gene expression assays for Gapdh (Mm99999915_g1), Cxcr4 (Mm01292123_m1) Cxcr5 (Mm00432086_m1), Cxcl12 (Mm00445553_m1) and Cxcl13 (Mm00444534_m1), and 1 ng/μL cDNA to a total volume of 15 μL per well. Real time quantitative polymerase chain reaction (RT-qPCR) was performed in a i-Cycler (Bio-Rad) connected to a QIOptics optical system (Bio-Rad) starting with denaturation for 10 minutes at 95°C followed by 45 cycles containing one 15 seconds step at 95°C and another at 60°C for 1 minute. Data were normalized to the reference gene Gapdh and presented as relative to the control.

2.9 | Bioinformatics analysis

Published data set of 13 055 cells (GEO: GSE109447) was used for the analysis. The single-cell RNA-Seq data were analyzed using the Seurat package (Version 3.0.1) in R computational environment (Version 3.6.0). We included genes detected in at least 10 cells for the analysis, and cells from male lateral SVZ with at least 100 and no more than 2500 genes expressed, at least 500 and no more than 4000 unique molecule identifiers (UMIs) and with less than 3% of total UMIs from mitochondrial genes (1374 cells retained in analysis). Data were log-normalized and top 500 highly variable genes (HVGs) were identified with the variance stabilizing transformation (VST) method. Total UMI count and percent mitochondrial contamination were regressed out during the gene scaling step. Principal component analysis was run on the HVGs and first 10 principal components were used for clustering and visualization using uniform manifold approximation and projection (UMAP). Default clustering algorithm in Seurat was used with resolution parameter set to 1. UMAP embedding was calculated with 21 neighbors (min_dist = 0.01, spread = 20).

2.10 | Data presentation and statistical analysis

All data were analyzed using GraphPad prism v7, and are presented as mean ± SEM. Shapiro-Wilk’s test was used to test all data for normal distribution. Data that passed the Shapiro-Wilk’s test were analyzed using a two-tailed unpaired t test to compare different groups and F test to test variance. Data that did not pass the Shapiro-Wilk’s test were analyzed using Mann-Whitney’s test. Significance was set to P < .05.

3 | RESULTS

3.1 | Neurogenesis is unaffected in the adult Cxcr5−/− SVZ

A previous study described that loss of Cxcr5 led to increased number of immature neural cells and decreased proliferation in the adult DG. However, it is not clear whether CXCL13/CXCR5 signaling also regulates SVZ neurogenesis. We therefore analyzed SVZ proliferation in 3-month-old WT and Cxcr5−/− mice. Animals were injected with ethynyl deoxyuridine (EdU), to label newly formed cells, four times over 1 day and sacrificed the same day. Immunolabeled EdU+ cells in the SVZ were then quantified in coronal brain sections. In contrast to previous findings in the DG, we did not detect any difference between WT and Cxcr5−/− mice in number of EdU+ cells in the SVZ (Figure 1A). Next, we examined cell populations involved in SVZ neurogenesis. A broad range of NSPCs express Sox2, including the stem cell population, while Mash1 more specifically labels TAPs, and DCX is a neuroblast specific marker. We therefore quantified total number of Mash1+, SOX2+, and DCX+ cells in brain sections of the SVZ and number of DCX+ cells in the OB from 3-month-old Cxcr5−/− and control animals using immunohistochemistry. However, we could not detect any difference between groups in any of the markers analyzed neither in the SVZ nor in the OB (Figure 1B-E). We also analyzed the formation of BrdU+/NeuN+ new mature neurons in the OB of animals that had received BrdU injections twice daily for 2 weeks and sacrificed 4 weeks after the last injection. As expected we could not detect any difference between WT and Cxcr5−/− mice (Figure 1F).

Our findings show that, in contrast to what have been reported for the DG, loss of Cxcr5 does not affect SVZ/OB neurogenesis in adult mice.

3.2 | Germline loss of Cxcr5 leads to increased SVZ proliferation in the aged brain

Given the close relationship between inflammation and neurogenesis, the increased inflammation in aged brain together with increased CXCL13 levels during brain inflammation we hypothesized that loss of Cxcr5 affect neurogenesis in the aged brain.

We first asked whether CXCR5/CXCL13 expression is regulated during aging. To assess this, we analyzed expression of Cxcr5 and Cxcl13 in adult (3 months) and aged (24 months) WT whole brains by RT-qPCR. Expression of Cxcr5 was very low and did not reveal any difference between adult and aged mice (Figure 2A). However, we did detect significantly higher expression of Cxcl13 in aged as compared to adult brains (Figure 2A), indicating a potential increase in CXCR5/CXCL13 signaling during aging, which could possibly affect neurogenesis.

To assess neurogenesis, we next analyzed SVZ proliferation in 18-month-old WT and Cxcr5−/− mice. Animals were injected with bromo deoxyuridine (BrdU), to label newly formed cells, four times over 1 day and sacrificed the same day. Immunolabeled BrdU+ cells in
the SVZ were then quantified in coronal brain sections. Interestingly, we observed a 1.6-fold increase in the amount of BrdU+ cells in SVZ of Cxcr5−/− mice as compared to controls (Figure 2B). We ruled out that major alterations to the SVZ cytoarchitecture were responsible for the changes in proliferation as no obvious change in the pattern of immunohistochemical staining was observed and the volume of the SVZ, as determined by Hoechst staining, was unchanged between Cxcr5−/− and control animals (Figure S1A,B).

NSPCs are the main pool of proliferating cells in the SVZ. To analyze whether the increased proliferation observed was due to a larger

![Diagram of BrdU+ cells in SVZ with quantification](image)

**FIGURE 1** Adult SVZ neurogenesis is not affected by loss of Cxcr5. Quantification and representative (overview and high magnification) images of, A, BrdU+, B, DCX+, C, Mash1+, and, D, SOX2+ cells, in the SVZ of 3-month-old wild type (WT) and Cxcr5−/− mice. Quantification and representative (overview and high magnification) images of, E, DCX+ and, F, BrdU+/NeuN+ cells in OB of 3-month-old WT and Cxcr5−/− mice. A-D, Data presented as means per 30 μm sections and, E,F, mean cell density (cells/mm³) per 20 μm section ± SEM; n = 8 (A-E), Cxcr5−/− n = 5, WT n = 6 (F). Scale bar = 100 μm (overview), 10 μm (high magnification). OB, olfactory bulb; SVZ, subventricular zone.
or more proliferative NSPC population, we next examined the NSPC compartment in the SVZ of WT and \( \text{Cxcr5}^{-/-} \) mice. We quantified number (Mash1+ and Sox2+) and proliferation (BrdU+/Mash1+ and BrdU+SOX2+ normalized to total Mash1+ and Sox2+, respectively) of NSPCs and TAPs in brain sections of the SVZ from \( \text{Cxcr5}^{-/-} \) and control animals using immunohistochemistry. However, we could not detect any difference in total number (Figure 2C,D) or proliferative capacity in either population (Figure 3A-C). These findings were
further supported by an in vitro neurosphere assay, where number and size of primary neurospheres is an indirect measure of amount and activity of NSPCs, which did not reveal any difference between WT and Cxcr5−/− mice (Figure 2E).

Taken together, our findings indicate that CXCR5/CXCL13 signaling is upregulated during aging and that loss of Cxcr5 leads to increased SVZ proliferation in the aged brain, which is not due to expanded or more proliferative NSC or TAP populations.

3.3 | Number of neuroblasts decreases in SVZ despite higher proliferation and is accompanied by neuroblast accumulation in the RMS and OB of aged Cxcr5−/− mice

NSCs and TAPs are not the only proliferating populations important for neurogenesis in the SVZ, but also neuroblasts have proliferative potential.19 To assess whether the increased SVZ proliferation observed could be due to altered neuroblast proliferation, we performed immunohistochemistry, colabeling BrdU with the neuroblast specific marker DCX and quantified double positive DCX+/BrdU+ cells in the SVZ of animals injected with BrdU over 1 day. Interestingly, we observed a higher ratio of double positive cells out of total DCX+ cells in the Cxcr5−/− mice as compared to controls (Figure 4A). This indicates that neuroblast proliferation increase in the SVZ of aged Cxcr5−/− mice and explains at least partly the overall higher SVZ proliferation observed. We speculated that the increased neuroblast proliferation might have an effect on the total population of neuroblasts in the SVZ, so we next quantified total number of DCX+ cells in sagittal RMS and coronal OB brain sections, and observed a threefold and twofold increase of neuroblasts in the RMS and OB, respectively, of Cxcr5−/− mice (Figure 4C,D). We also observed that the largest difference in number of DCX+ cells occurred in the RMS closest to the OB (Figure S2A,B). Neuroblasts continue to divide as they migrate through the RMS.20 Therefore, we wanted to analyze if changes in proliferation in the RMS could contribute to the increased density of neuroblasts observed. We quantified the total amount of double positive DCX+/BrdU+ over total DCX+ cells, and detected a twofold increase in the Cxcr5−/− mice as compared to controls (Figure 4C).

To rule out that the decreased amount of neuroblasts in the SVZ and increased amount in the RMS and OB of Cxcr5−/− mice is due to alteration in cell death, we analyzed the rate of apoptosis using a TUNEL assay. We could not detect any difference in the total number of apoptotic TUNEL+ cells in the SVZ, RMS, or OB in Cxcr5−/− as compared to WT mice (Figure S2C-E).

In conclusion, our findings suggest that loss of Cxcr5 increases neuroblast proliferation and increases their migration out of the SVZ leading to lower number in the SVZ but increased numbers in the later parts of RMS and in the OB.

3.4 | Number of newly formed neurons remains unchanged in the OB

After migrating through the RMS, neuroblasts, under normal conditions, reach the OB where they differentiate into interneurons.21 We next asked whether the increased amount and proliferation of neuroblasts in the RMS and OB translates to increased number of new neurons in the OB. To assess formation of new neurons, mice were injected with BrdU 2 times per day over 2 weeks, and sacrificed 4 weeks after the last injection. OB sections from aged Cxcr5−/− and
FIGURE 4  Loss of Cxcr5 increase neuroblast proliferation and migration. A, Quantification (left) and representative images (middle) of DCX+/BrdU+ cells in SVZ of 18-month-old WT and Cxcr5−/− mice, and orthogonal projection confirming BrdU colocalization with DCX (right). B, Quantification and representative (overview and high magnification) images of number of DCX+ cells in SVZ of WT and Cxcr5−/− mice. C, Quantification and example (overview and high magnification) images of DCX+ and BrdU+/DCX+ neuroblasts in sagittal sections from the center of the RMS of 18-month-old WT and Cxcr5−/− mice. D, Quantification and representative (overview and high magnification) images of number of DCX+ cells in OB of 18-month-old WT and Cxcr5−/− mice. A,C, Data presented as mean percentage per 30 μm sections; B,C, mean number per 30 μm sections; and, D, mean cell density (cells/mm²) per 20 μm section ± SEM; n = 5 (A, B), Cxcr5−/− n = 5, WT n = 4 (C), n = 4 (D). *P < .05, with two-tailed unpaired t test. Scale bar = 50 μm (A [middle]), 20 μm (A [right]), 100 μm (B, D [overview]), 300 μm (C [overview]), 10 μm (B, C, D [high magnification]). OB, olfactory bulb; RMS, rostral migratory stream; SVZ, subventricular zone; WT, wild type

FIGURE 5  Number of newly formed neurons in the OB is not affected by loss of Cxcr5. A, Total number (left), percentage (middle), and representative images (right) of NeuN+/BrdU+ cells in the OB (GCL and IPL) of 18-month-old WT and Cxcr5−/− mice. B, Orthogonal projection confirming BrdU colocalization with NeuN. A, Data presented as mean cell density (cells/mm²) per 20 μm section and, B, mean percentage per 20 μm section ± SEM; n = 9 (A), Cxcr5−/− n = 9, WT n = 5. *P < .05, with two-tailed unpaired t test. Scale bar = 100 μm (A), 20 μm (B). GCL, granule cell layer; IPL, internal plexiform layer; OB, olfactory bulb; WT, wild type
FIGURE 6  Loss of Cxcr5 leads to increased levels of circulating IL-6, and Cxcl12 expression in SVZ. A, Cytometric bead array analysis of circulating cytokine concentrations in plasma from 20-month-old wild type (WT) and Cxcr5−/− mice. B, mRNA expression as assessed by real time quantitative polymerase chain reaction (RT-qPCR) of Il-6 in SVZ tissue from 20- to 22-month-old WT and Cxcr5−/− mice. C, Quantification (left) and representative images (middle) of, IBA1+, and percentage of activated, IBA1+/ED1+ microglia in the SVZ of 18-month-old WT and Cxcr5−/− mice. Orthogonal projection confirming colocalization of IBA1 and ED1 (right). D, Quantification of microglia activation by morphometric analysis of Iba1 + microglia, from ramified-, through intermediate, to amoeboid- and round morphology. E, mRNA expression as assessed by RT-qPCR of Cxcr4 and Cxcl12 in SVZ tissue from 20- to 22-month-old WT and Cxcr5−/− mice. A, Data presented as mean concentration (pg/mL plasma); B,E, mean fold change to Gapdh; C, mean per 30 μm sections; and C,D, mean ratio (%) per 30 μm sections ± SEM; n = 4 (A), n = 3 (B, E), n = 5 (C, D). *P < .05, **P < 0.01 with two-tailed unpaired t test. Scale bar = 50 μm (A [middle]), 20 μm (A [right]). SVZ, subventricular zone.
control animals were prepared and analyzed using immunohistochemistry. We quantified the total number of BrdU+ and BrdU+/NeuN+ double positive cells in the GCL and IPL and calculated the ratio normalized to total number of BrdU+ cells. We did not detect any changes in total number of double positive cells and only a modest increase in the ratio of newly formed neurons in Cxcr5−/− mice as compared to controls (Figure 5A,B).

These findings show that the increased total number and proliferation of neuroblasts in the RMS and OB does not increase the total amount of new mature neurons in the OB.

3.5 | Decreased number of SVZ microglia and altered cytokine expression in aged Cxcr5−/− mice

We next sought to investigate possible mechanisms that could explain the altered neuroblast proliferation and migration observed. We first examined the expression of Cxcr5 in SVZ tissue and sorted GFP+ neuroblasts from SVZ of Dcx-GFP mice using RT-qPCR. Surprisingly, expression of Cxcr5 was undetectable (data not shown). However, RT-qPCR in bulk populations and tissue might not detect expression if a discrete population of cells expresses the gene of interest. To determine Cxcr5 and Cxcl13 expression on a single cell level in all relevant cells of the SVZ, we reanalyzed a previously published single cell RNAseq data set including NSPCs, neuroblasts, endothelial cells, mural cells, fibroblasts, astrocytes, neurons, oligodendrocytes, and ependymal cells from the SVZ. Strikingly, this analysis revealed that Cxcr5 and Cxcl13 are undetectable in all cell types in the SVZ (Figure S3A-C). Taken together, these results strongly indicate that the effect on neuroblasts is indirect.

Given the role of CXC5 in immune cells and that inflammation is a potent regulator of neurogenesis, we next explored if inflammation is altered in aged Cxcr5−/− mice and thereby could constitute an indirect action on neurogenesis. In a previous study, the amount of circulating pro and anti-inflammatory cytokines (IFNy, TNF, IL-12p70, IL-10, MCP-1, IL-6) was assessed in young adult mice using a cytometric bead array, without detecting any differences. We used the same technique to reveal any gross alterations of inflammation in aged Cxcr5−/− mice. Interestingly, we detected a significant increase of IL-6 in plasma from Cxcr5−/− mice compared to control mice, but otherwise no differences could be observed (Figure 6A). Because IL-6 has been shown to increase proliferation of NSPCs, we analyzed IL-6 expression also in SVZ tissue from WT and Cxcr5−/− mice by RT-qPCR but could not detect any difference (Figure 6B) indicating that the elevated level of IL-6 in circulation is not present in the SVZ.

In the brain, microglia are mediators of inflammation and effect different stages of neurogenesis. Therefore, we decided to investigate if loss of Cxcr5 had any influence on number and activation of microglia in the SVZ. We first quantified total amount of microglia in the SVZ. Interestingly, we detected a significant increase in adult but decrease in aged Cxcr5−/− mice in the number of IBA1+ microglia as compared to age-matched controls (Figure 6C and Figure S4A). We next examined the activation status of microglia in the SVZ. Microglia activation can be assessed by the coexpression of ED1 and the progressive change from ramified, to amoeboid- and finally round morphology. We therefore compared the number of activated, double positive IBA1+/ED1+ microglia normalized to total amount of IBA1+ microglia, as well as morphology of IBA1+ microglia. However, we did only detect a modest increase of ramified microglia in adult Cxcr5−/− mice and no difference in any of these parameters in aged Cxcr5−/− mice (Figures 5A/B and 5C/D).

We could not detect any expression of Cxcr5 on sorted neuroblasts from the SVZ of Dcx-GFP mice (data not shown). However, it has been reported that both NSPCs and DCX+ neuroblasts express Cxcr4, another member of the CXC family of chemokine receptors. Neuroblasts use Cxcr4 and its ligand CXCL12 for migration in the brain and CXCL12 has been suggested to promote proliferation of Cxcr4 expressing cells. Therefore, we decided to investigate if lack of Cxcr5 could lead to changes in the Cxcr4/Cxcl12 pathway and potentially explain the specific effects observed on neuroblasts. First, we analyzed the normal expression pattern of Cxcr4 and Cxcl12 in the SVZ by bioinformatics analysis of the same single cell RNAseq data set described earlier. This analysis revealed, in agreement with previous studies, that Cxcl12 is mainly expressed in endothelial cells while Cxcr4 was expressed in NSPCs, including neuroblasts. However, both Cxcl12 and Cxcr4 were also to some extent expressed in other SVZ cells (Figure S3D,E). Next, we analyzed expression in SVZ tissue using RT-qPCR. Although we were not able to detect any differences in Cxcr4 expression, we did, however, observe higher expression of Cxcl12 in the SVZ of aged Cxcr5−/− mice as compared to controls (Figure 6E).

Taken together, these findings demonstrate that loss of Cxcr5 increases circulating IL-6 in aged blood, decreases the amount of IBA1+ microglia in the aged SVZ without affecting their activation status, and increases expression of Cxcl12 in the aged SVZ offering a possible mechanism explaining the increased proliferation and lower number of neuroblasts in the aged SVZ.

4 | DISCUSSION

We show here for the first time that CXCR5/CXCL13 signaling alters neurogenesis in the aged murine SVZ and we suggest a mechanism mediated through a combination of altered levels of systemic and local inflammatory mediators.

We observed increased proliferation but lower total number of neuroblasts in the SVZ of aged Cxcr5−/− as compared to control mice. However, the increased neuroblast proliferation did not translate into more new mature neurons in the OBs. Instead, neuroblasts accumulated in the RMS and OB, indicating that neuroblasts in Cxcr5−/− mice migrated out of the SVZ more efficiently as compared to in control animals. The fact that the increased number of neuroblasts in the RMS and OB did not translate into more new mature neurons in the OB is puzzling. One potential explanation could be that the surplus of neuroblasts blocks efficient integration. Perhaps a more likely explanation is that...
neuroblasts in Cxcr5−/− mice favor proliferation rather than maturation as proliferation has been shown to inhibit neuronal differentiation.30

The observed changes were specific to the neuroblast population and did not affect other neurogenic cells, such as NSCs or TAPs.

Previous studies have shown higher expression of Cxcl13 in aged peripheral lymph nodes.31 and increased CXCL13 levels during brain inflammation,11,32 supporting our discovery of higher Cxcl13 expression in the aged brain. Given this increased expression, it is tempting to speculate that Cxcr5 signaling is at least partly responsible for the observed reduced neuroblast proliferation33 in the aged SVZ.

Our observation that loss of Cxcr5 affects neurogenesis is supported by previous findings where knockout of Cxcr5 affected neurogenesis in the adult murine DG. However, this study observed rather opposite effects with decreased proliferation and increased number of progenitors.13 This discrepancy is most likely due to differences between the different neurogenic niches (SVZ vs DG) and age groups (adult vs aged) studied. For instance, SVZ and DG have different architecture and milieu, for example, NSCs in the SVZ have direct contact with the lateral ventricle and CSF which DG neural stem cells do not have.34 Similarly, there are clear differences in the cell composition and transcriptome between aged and adult neurogenic niches.35

We did not detect any changes in SVZ neurogenesis in adult mice, indicating that loss of Cxcr5 affects neurogenesis in a region and age specific manner.

Further supporting our findings, Cxcr5 was shown to regulate proliferative capacity of radial glial cells (RGCs) and neuronal differentiation during regeneration in the adult zebrafish brain.12 In contrast to findings from zebrafish, where progenitor RGCs were shown to express Cxcr5, we did not detect any expression of Cxcr5 in sorted neuroblasts or any other SVZ cells. This strongly suggests that the effect observed is indirect and mediated through other cells in the brain such as microglia or altered inflammation locally or at the systemic level.

We detected higher number of microglia in the adult but lower number in the aged Cxcr5−/− SVZ without any changes in activation, neither by morphological analysis nor by expression of the activated microglia marker ED1. Without differences in microglia activation, it is unlikely that acutely altered inflammation in the aged SVZ is responsible for the alterations in the neuroblast population. We can, however, not exclude that changes in inflammation or the reduction in number of microglia, occurring during the course of aging in absence of Cxcr5, could affect neurogenesis.

Although we did not observe any signs of altered microglia activation in the aged SVZ, we found higher levels of IL-6 in plasma from aged Cxcr5−/− mice. IL-6 is mainly produced by immune regulating cells such as monocytes,36 systemic IL-6 can enter CSF as well as the brain parenchyma through the BBB in adult mice37,38 and therefore have the potential to reach the SVZ. Interestingly, IL-6 has been suggested to induce CXCL12 mediated chemotaxis and proliferation in astroglia by upregulating Cxcr4.39 However, since we could not detect any difference in Cxcr4 expression in SVZ tissue, it is unlikely that the altered migration observed is due to IL-6 mediated increase in Cxcr4 expression. On the other hand, IL-6 has been shown to promote proliferation of neuroblasts in the adult SVZ.40 Importantly, systemically delivered IL-6 has been shown to effect neurogenesis by direct action on SVZ cells.41 This indicates that the elevated level of circulating IL-6 in Cxcr5−/− mice is at least partly responsible for the altered proliferation observed. Interestingly, in adult Cxcr5−/− mice where we did not observe any differences in SVZ proliferation, plasma levels of IL-6 were also indistinguishable from WT controls.13

Another possible reason for the changes in both proliferation and migration of neuroblasts is the increased level of Cxcl12 that we observed in the SVZ of Cxcr5−/− mice. Cxcl12 is expressed by endothelial cells and neuronal precursors in SVZ, and has been shown to promote proliferation and migration of neuroblasts.52 Supporting the idea of Cxcl12 as responsible for the increased migration out of the SVZ, Cxcl12 have been shown to increase migration of T-cells and HeLa cells even in the absence of a concentration gradient.43,44 Therefore, a plausible explanation for our findings is that loss of Cxcr5 leads to increased systemic levels of IL-6, which together with increased Cxcl12 expression in the SVZ promote neuroblast proliferation and their migration out of the SVZ.

5 | CONCLUSION

Here, we provide the first experimental evidence that germline loss of Cxcr5 specifically alters neuroblast proliferation and migration in the aged brain and that this might occur through systemic and local increase of the inflammatory mediators IL-6 and CXCL12. It now seems highly warranted to explore the interplay between other circulating and local immunomodulatory factors in regulating the neurogenic decline during aging. The present study is important also from a clinical translational perspective since blocking CXCR5/CXCL13 signaling has been proposed as a therapeutic strategy in neuroinflammation.53 Our findings raise the possibility that long-term inhibition of neuroinflammation by reducing CXCR5/CXCL13 signaling might compromise neurogenesis in older patients.

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CONFLICT OF INTEREST

The authors declared no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

J.F.: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript; A.G.: collection and assembly of data, data analysis and interpretation, final approval of manuscript; R.M., E.Q., E.S.: collection

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and assembly of data, final approval of manuscript; E.M.: assembly of data, final approval of manuscript; P.D., S.L.: data analysis and interpretation, final approval of manuscript; H.A.: conception and design, financial support, administrative support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author.

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REFERENCES
1. Lim DA, Alvarez-Buylla A. The adult ventricular-subventricular zone (V-SVZ) and olfactory bulb (OB) neurogenesis. Cold Spring Harb Perspect Biol. 2016;8(5).
2. Kempermann G, Song HJ, Gage FH. Neurogenesis in the adult hippocampus. Cold Spring Harb Perspect Biol. 2015;7(9).
3. Ahlenius H, Visan V, Kokaia M, Lindvall O, Kokaia Z. Neural stem and progenitor cells retain their potential for proliferation and differentiation into functional neurons despite lower number in aged brain. J Neurosci. 2009;29(14):4408-4419.
4. Nunez-Parra A, Pugh V, Araneda RC. Regulation of adult neurogenesis by behavior and age in the accessory olfactory bulb. Mol Cell Neurosci. 2011;47(4):274-285.
5. Mobley AS, Bryant AK, Richard MB, Brann JH, Firestein SJ, Greer CA. Age-dependent regional changes in the rostral migratory stream. Neurol Biol. 2013;34(7):1873-1881.
6. Elaby M, Jackaman C, Mamo J, et al. Blood-brain barrier dysfunction developed during normal aging is associated with inflammation and loss of tight junctions but not with leukocyte recruitment. Immun Ageing. 2015;12:2.
7. Spitta B. Aging microglia—phenotypes, functions and implications for age-related neurodegenerative diseases. Front Aging Neurosci. 2017;9:194.
8. Solano Fonseca R, Mahesula S, Apple DM, et al. Neurogenic niche microglia undergo positional remodeling and progressive activation contributing to age-associated reductions in neurogenesis. Stem Cells Dev. 2016;25:542-555.
9. Schafer DP, Stevens B. Microglia function in central nervous system development and plasticity. Cold Spring Harb Perspect Biol. 2015;7(10):a020945.
10. Jiang BC, Cao DL, Zhang X, et al. CXCL13 drives spinal astrocyte activation and neuropathic pain via CXCR5. J Clin Investig. 2016;126(2):745-761.
11. Krumholz M, Theil D, Cepok S, et al. Chemokines in multiple sclerosis: CXCL12 and CXCL13 up-regulation is differentially linked to CNS immune cell recruitment. Brain. 2006;129:200-211.
12. Kızıl C, Dudçizg S, Kırktısı N, et al. The chemokine receptor ccr5 regulates the regenerative neurogenesis response in the adult zebrafish brain. Neural Dev. 2012;7:27.
13. Stuart MJ, Corrigan F, Baune BT. Knockout of CXCR5 increases the population of immature neural cells and decreases proliferation in the hippocampal dentate gyrus. J Neuroinflammation. 2014;11:31.
14. Thore P, Heldmann U, Gomes-Leal W, et al. Long-term accumulation of microglia with proneurogenic phenotype concomitant with persistent neurogenesis in adult subventricular zone after stroke. Glia. 2009;57(8):835-849.
15. Ahlenius H, Kokaia Z. Isolation and generation of neurosphere cultures from embryonic and adult mouse brain. Methods Mol Biol. 2010; 633:241-252.
16. Mizrak D, Levitin HM, Delgado AC, et al. Single-cell analysis of regional differences in adult V-SVZ neural stem cell lineages. Cell Rep. 2019;26(2):394-406.
17. Ekdahl CT, Claassen JH, Bonde S, Kokaia Z, Lindvall O. Inflammation is detrimental for neurogenesis in adult brain. Proc Natl Acad Sci USA. 2003;100(23):13632-13637.
18. Bektas A, Schurman SH, Sen R, Ferrucci L. Aging, inflammation and the environment. Exp Gerontol. 2018;105:10-18.
19. Kuhn HG, Winkler J, Kempermann G, Thal LJ, Gage FH. Epidermal growth factor and fibroblast growth factor-2 have different effects on neural progenitors in the adult rat brain. J Neurosci. 1997;17(15): 5820-5829.
20. Ikeda M, Hirota Y, Sakaguchi M, et al. Expression and proliferation-promoting role of diversin in the neuronally committed precursor cells migrating in the adult mouse brain. Stem Cells. 2010;28(11):2017-2026.
21. Alvarez-Buylla A, Garcia-Verduco JM. Neurogenesis in adult subventricular zone. J Neurosci. 2002;22(3):629-634.
22. Zonis S, Ljubimov VA, Mahgerefteh M, Pechnick RN, Wawrowsky K, Chesnokova V. p21Cip restrains hippocampal neurogenesis and protects neuronal progenitors from apoptosis during acute systemic inflammation. Hippocampus. 2013;23(12):1383-1394.
23. Borsini A, Zunzszan PA, Thuret S, Pariante CM. The role of inflammatory cytokines as key modulators of neurogenesis. Trends Neurosci. 2015;38(3):145-157.
24. Goings GE, Koslowski DA, Szele FG. Differential activation of microglia in neurogenic versus non-neurogenic regions of the forebrain. Glu. 2006;54(4):329-342.
25. Tran PB, Banisadr G, Ren D, Chenn A, Miller RJ. Chemokine receptor expression by neural progenitor cells in neurogenic regions of mouse brain. J Comp Neurol. 2007;500(6):1007-1033.
26. Filippo TRM, Galindo LT, Barnabe GF, et al. CXCL12 N-terminal end is sufficient to induce chemotaxis and proliferation of neural stem/progenitor cells. Stem Cells Dev. 2013;22(11):913-925.
27. Kokovay E, Goderie S, Wang Y, et al. Adult SVZ lineage cells home to and leave the vascular niche via differential responses to SDF1/CXCR4 signaling. Cell Stem Cell. 2010;7(2):163-173.
28. Barkho BZ, Munoz AE, Li X, Li L, Cunningham LA, Zhao X. Endogenous matrix metalloproteinase (MMP)-3 and MMP-9 promote the differentiation and migration of adult neural progenitor cells in response to chemokines. Stem Cells. 2008;26(12):3139-3149.
29. Li M, Ransohoff RM. Multiple roles of chemokine CXCL12 in the central nervous system: a migration from immunology to neurobiology. Prog Neurobiol. 2008;84(2):116-131.
30. Hardwick LJ, Ali FR, Azzarelli R, Philpott A. Cell cycle regulation of proliferation versus differentiation in the central nervous system. Cell Tissue Res. 2015;359(1):187-200.
31. Becklund BR, Purton JF, Ramsey C, et al. The aged lymphoid tissue environment fails to support naive T cell homeostasis. Sci Rep. 2016; 6:30842.
32. Chapman KZ, Ge R, Monni E, et al. Inflammation without neuronal death triggers striatal neurogenesis comparable to stroke. Neurobiol Dis. 2015;83:1-15.
33. Tropeva V, Craig CG, Morshed CM, van der Kooy D. Transforming growth factor-alpha null and senescent mice show decreased neural progenitor cell proliferation in the forebrain subependyma. J Neurosci. 1997;17(20):7850-7859.
34. Ming GL, Song H. Adult neurogenesis in the mammalian brain: significant answers and significant questions. Neuron. 2011;70(4): 687-702.
35. Smith LK, White CW, Villeda SA. The systemic environment: at the interface of aging and adult neurogenesis. Cell Tissue Res. 2018;371(1):105-113.
36. Naka T, Nishimoto N, Kishimoto T. The paradigm of IL-6: from basic science to medicine. Arthritis Res. 2002;4(suppl 3):S233-S242.
37. Banks WA, Kastin AJ, Broadwell RD. Passage of cytokines across the blood-brain barrier. *Neuroimmunomodulation*. 1995;2(4):241-248.
38. Banks WA, Kastin AJ, Gutierrez EG. Penetration of interleukin-6 across the murine blood-brain-barrier. *Neurosci Lett*. 1994;179(1-2):53-56.
39. Odemis V, Moepps B, Gierschik P, Engele J. Interleukin-6 and cAMP induce stromal cell-derived factor-1 chemotaxis in astroglia by up-regulating CXCR4 cell surface expression. Implications for brain inflammation. *J Biol Chem*. 2002;277(42):39801-39808.
40. Bowen KK, Dempsey RJ, Vemuganti R. Adult interleukin-6 knock-out mice show compromised neurogenesis. *Neuroreport*. 2011;22(3):126-130.
41. Storer MA, Gallagher D, Fatt MP, Simonetta JV, Kaplan DR, Miller FD. Interleukin-6 regulates adult neural stem cell numbers during normal and abnormal post-natal development. *Stem Cell Reports*. 2018;10(5):1464-1480.
42. Cheng X, Wang H, Zhang X, et al. The role of SDF-1/CXCR4/CXCR7 in neuronal regeneration after cerebral ischemia. *Front Neurosci*. 2017;11:590.
43. Pelletier AJ, van der Laan LJW, Hildbrand P, et al. Presentation of chemokine SDF-1 alpha by fibronectin mediates directed migration of T cells. *Blood*. 2000;96(8):2682-2690.
44. Dillenburg-Pilla P, Patel V, Mikulis CM, et al. SDF-1/CXCL12 induces directional cell migration and spontaneous metastasis via a CXCR4/Galpha(i)/mTORC1 axis. *FASEB J*. 2015;29(3):1056-1068.
45. Huber AK, Irani DN. Targeting CXCL13 during neuroinflammation. *Adv Neuroimmune Biol*. 2015;6(1):1-8.

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