Deciphering Age-Related Decline in Neurogenesis. Focusing on Intermediate Progenitors and Their Reaction to Immune Signalling

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Focusing on Intermediate Progenitors and Their Reaction to Immune Signalling

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LUND
UNIVERSITY

DOCTORAL DISSERTATION

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Key words: Neurogenesis, Aging, Subventricular Zone (SVZ), Dentate Gyrus (DG), Inflammation, Intermediate Progenitors (IPs), Microglia, CX3CR1, CXCR5, Neuroblasts

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Artistic interpretation of a newly generated neuron with aged characteristics emerging from the neurogenic niche of an aged brain, as immune cells both inside and outside the niche influence its development and migration.

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Relax, and live longer

(but don't forget to exercise)

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II. **Fritze, J**., C. Muralidharan, E. Stamp, and H. Ahlenius. (2024). 'Microglia undergo disease-associated transcriptional activation and CX3C motif chemokine receptor 1 expression regulates neurogenesis in the aged brain', *Developmental Neurobiology*.

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Abstract

Neurogenesis continues throughout life in two key regions, the subventricular zone (SVZ) and dentate gyrus (DG), but declines with age alongside increased chronic inflammation and microglial activation. These neurogenic niches differ in their susceptibility to systemic changes, with the SVZ located near cerebrospinal fluid and the DG influenced by local neural activity. We found that immune changes, including altered expression of microglia-specific *Cx3cr1*, leukocyte-specific *Cxcr5*, systemic cytokine IL-6, and local *Cxcl12*, specifically impact SVZ intermediate progenitors during aging, leading to distinct effects on neurogenesis in the SVZ compared to the DG. In addition, we identified a subset of SVZ neuroblasts that acquire an immune-related transcriptional profile during aging, indicating a potential role in how immune changes influence neurogenesis. Our findings highlight inflammation as a central driver of age-related neurogenic decline and emphasize the need for niche- and age-specific therapeutic strategies that target immune cells or signaling pathways.

Lay summary

Throughout our lives, new nerve cells are continuously generated in specific areas of the brain. Two important regions for this process are the subventricular zone (SVZ), located near the brain's fluid-filled ventricles, and the hippocampus (HC), a region crucial for memory. However, as we get older, this process of producing new neurons, called neurogenesis, slows down. This decline is closely related to increased inflammation in the brain and the activation of immune cells called microglia. While microglia usually protect the brain, they can cause harmful inflammation when they become active for too long.

The environments of the SVZ and HC are different. The SVZ, positioned near cerebrospinal fluid, is more affected by changes in the body's immune system, while the HC is more influenced by local brain activity. This suggests that aging may impact these two regions in different ways.

In our study, we found that changes in the immune system both in the brain and outside the brain particularly affect the production of new neurons in the SVZ, differently than in the HC during aging.

We also identified a group of stem cell like cells in the SVZ that react strongly to inflammation, which could interfere with their ability to develop into fully functional neurons.

In summary, our research reveals that inflammation is a significant driver of the age-related decline in neurogenesis. It also underscores the importance of treatments that consider how different regions of the brain respond to aging and inflammation. By targeting specific immune cells or molecules, it may be possible to slow down or even reverse some of the negative effects of aging on the brain's ability to produce new neurons.

Populärvetenskaplig sammanfattning

Under hela livet bildas nya nervceller i specifika områden i hjärnan. Två viktiga områden för denna process är den subventrikulära zonen (SVZ), som ligger nära hjärnans vätskefyllda ventriklar, samt hippocampus (HC), en region som är avgörande för minnet. Men när vi blir äldre avstannar denna process, som kallas neurogenes. Denna nedgång är starkt kopplad till ökad inflammation i hjärnan och aktivering av immunceller i hjärnan som kallas mikroglia. Även om mikroglia vanligtvis skyddar hjärnan, kan de orsaka skadlig inflammation om de är aktiva för länge.

Områdena i SVZ och HC skiljer sig åt. SVZ, som finns nära cerebrospinalvätskan, påverkas mer av förändringar i kroppens immunsystem, medan DG påverkas mer av lokal hjärnaktivitet. Detta tyder på att åldrandet kan påverka dessa två regioner på olika sätt.

I vår studie fann vi att förändringar i immunsystemet både i och utanför hjärnan särskilt påverkar produktionen av nya nervceller i SVZ på ett annorlunda sätt än i HC under åldrandet.

Vi identifierade också en grupp stamcellsliknande celler i SVZ som påverkas extra mycket av inflammation när de åldras, vilket kan störa deras förmåga att utvecklas till fullt fungerande nervceller.

Sammanfattningsvis visar vår forskning att inflammation är en betydande drivkraft för den åldersrelaterade minskningen av nybildning av nervceller. Den understryker också vikten av behandlingar som tar hänsyn till hur olika delar av hjärnan reagerar på åldrande och inflammation. Genom att rikta in sig på specifika immunceller eller molekyler kan det vara möjligt att bromsa eller till och med vända några av de negativa effekterna av åldrande på hjärnans förmåga att producera nya nervceller.

Abbreviations

AMPK	AMP Activated Protein Kinase
Ascl1	Achaete-Scute Family BHLH Transcription Factor 1
BBB	Bood-Brain Barrier
BMP4	Bone Morphogenetic Protein 4
BrdU	Bromodeoxyuridine
bFGF	Basic Fibroblast Growth Factor
Calbindin	Calbindin-D28k
CNS	Central Nervous System
CSF	Cerebrospinal Fluid
Cx3cr1	CX3C Motif Chemokine Receptor 1
Cxcl12	CXC Motif Chemokine Ligand 12
Cxcl13	CXC Motif Chemokine Ligand 13
Cxcr4	CXC Chemokine Receptor 4
Cxcr5	CXC Chemokine Receptor 5
DABCO	1,4 Diazabicyclo[2.2.2]octane
DAM	Disease Associated Microglia
DAPI	4',6 diamidino 2 phenylindole
DCX	Doublecortin
DEGs	Differentially Expressed Genes
DGE	Differential Gene Expression
DG	Dentate Gyrus
Dlx2	Distal-Less Homeobox 2
ECM	Extracellular Matrix
EGF	Epidermal Growth Factor
FACS	Fluorescence Activated Cell Sorting
GABA	Gamma-Aminobutyric Acid
GCL	Granule Cell Layer

GFAP	Glial Fibrillary Acidic Protein
GFP	Green Fluorescent Protein
GO	Gene Ontology
HSC	Hematopoietic Stem Cell
IFN-γ	Interferon Gamma
Igfbpl1	Insulin Like Growth Factor Binding Protein Like 1
IL-6	Interleukin 6
IPL	Internal Plexiform Layer
IP	Intermediate Progenitor
Lgals9	Galectin 9
MACS	Magnetic Activated Cell Sorting
MCP-1	Monocyte Chemoattractant Protein 1
NeuN	Neuronal Nuclei (RBFOX3)
NeuroD1	Neuronal Differentiation 1
NSC	Neural Stem Cell
OB	Olfactory Bulb
P2ry12	Purinergic Receptor P2Y12
PCA	Principal Component Analysis
PFA	Paraformaldehyde
Prox1	Prospero Homeobox 1
PSA-NCAM	Polysialylated-Neural Cell Adhesion Molecule
qRT-PCR	Quantitative Reverse Transcription Polymerase Chain Reaction
RMS	Rostral Migratory Stream
RNA	Ribonucleic Acid
SGZ	Subgranular Zone
Shh	Sonic Hedgehog

Sox2	SRY-Box Transcription Factor 2
SVZ	Subventricular Zone
Tbr2	T box Brain Transcription Factor 2
TMEM119	Transmembrane Protein 119
TNF-α	Tumor Necrosis Factor Alpha
TSA	Tyramide Signal Amplification
UMAP	Uniform Manifold Approximation and Projection
USP18	Ubiquitin Specific Peptidase 18
VGlut1/2	Vesicular Glutamate Transporter 1/2
VZ	Ventricular Zone

Introduction

Neurogenesis

Neurogenesis, the formation of new neurons, primarily occurs during CNS development and in two regions of the adult mammalian brain; the subventricular zone (SVZ) lining the lateral ventricles, and the dentate gyrus (DG) that reside in the hippocampus (HC). In the SVZ, neural stem cells (NSCs) generate intermediate progenitors (IPs) that migrate to the olfactory bulb (OB) where they differentiate into mature neurons and contribute to olfactory function. In the DG, new neurons mature without leaving the region, and are crucial for learning, memory, and emotional regulation (Figure 1) [1, 2].

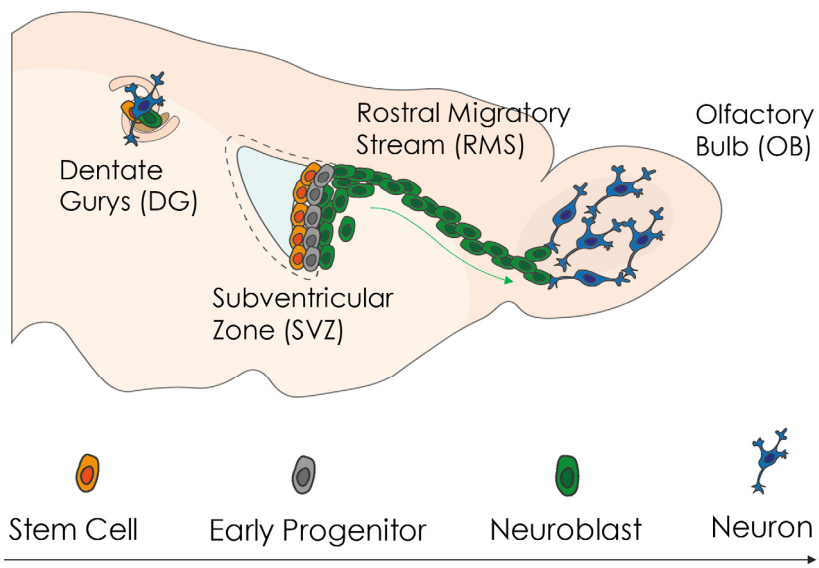

Figure 1 Schematic ilustratin of murine neurogenesis
Neural stem cells (NSCs) in the subventricular zone (SVZ) produce intermediate progenitors that differentiate into another intermediate state called neuroblasts which migrate along the rostral migratory stream (RMS) until they reach the olfactory bulb (OB) where they mature into interneurons. In the dentate gyrus (DG) NCS generate intermediate progenitors that differentiate and mature inte excitatory neurons in the same region.

Embryonic neurogenesis

During development, neurogenesis occurs in the neuroepithelium of the developing neural tube. Neuroepithelial cells proliferate in the ventricular zone (VZ) near the inner lumen (ventricle) to form the neural tube. These cells are arranged in a polarized, single-layered sheet, where the base contacts the basal lamina and the apical end touches the ventricle. Initially, symmetric cell division expands the neural epithelium and the pool of neural progenitors which then differentiate into various types of neurons and glial cells. [3, 4] Key signaling pathways, such as Notch, Wnt, and Sonic Hedgehog, play crucial roles in guiding proliferation, differentiation, and migration of nurogenic cells. [5-7]

Adult Neurogenic regions

SVZ and DG neurogenesis in the adult brain are both rooted in the early stages of embryonic neurogenesis. The SVZ originates from the lateral ganglionic eminence (LGE), a structure formed from proliferating cells in the VZ, while the DG arises from the medial ganglionic eminence (MGE) and the cortical neuroepithelium. As embryogenesis progresses, radial glial cells give rise to ependymal cells, which line the brain ventricles, and a subset of radial glia that remain as NSCs with radial glia-like properties. These NSCs are retained in both the SVZ and DG into adulthood, continuing to generate new neurons throughout life.

However, the timing and maturity if NSCs that shape the two neurogenic regions differ. In the SVZ, NSCs branch off from their radial glial lineage around embryonic day 14 (E14), entering quiescence and only becoming active during adulthood. In contrast, NSCs in the DG continue to develop during the postnatal period, adopting a unique transcriptomic identity between postnatal days 14 and 18 (P14-P18). The developmental origins of the SVZ and DG shape their neurogenic differences in adulthood, with SVZ NSCs having greater multipotency and generating inhibitory neurons, while DG NSCs produce excitatory neurons. [3, 4, 8-10]

Adult neurogenesis beyond the DG and SVZ/OB remains a subject of ongoing investigation and debate. Evidence suggests that neurogenesis may originate in other areas of the adult brain, such as the hypothalamus, striatum, amygdala, and certain regions of the cortex, although at significantly lower levels compared to the DG and SVZ [11, 12]. Several studies indicate the presence of neural progenitor cells and the generation of new neurons in these regions, particularly following injury or other non-homeostatic events [13-15]. However, this is distinct from neurogenesis driven by progenitors migrating from the main neurogenic regions, as seen in repair following

stroke, which is strongly supported by experimental evidence [16-19]. While neurogenesis outside the DG and SVZ is plausible, it likely contributes marginally to overall brain plasticity and repair. Further research is essential to definitively establish the presence, mechanisms, and potential roles of neurogenesis in these less conventional sites.

Dentate gyral neurogenesis

From a developmental perspective, the formation of the DG is unique, the region originates from a distinct progenitor cell source located near the pial surface, away from the ventricular zone (VZ). This specialized proliferative zone remains active throughout postnatal development and ultimately transforms into the subgranular zone (SGZ) [3, 11, 20].

Radial glia-like neural stem cells (Type 1 cells) reside in a quiescent state in the SGZ and possess long radial processes that extend into the molecular layer. These cells, when activated, have a slow proliferation rate and can differentiate into neurons through intermediate stages. Type 1 cells express markers such as GFAP, Nestin, and Sox2, which are characteristic of neural stem cells. As Type 1 cells progress into intermediate progenitor cells (Type 2a), they retain Nestin and Sox2 expression, while GFAP gradually declines. Type 2a cells are proliferative and exhibit transient amplifying properties. As these cells commit to a neuronal lineage (Type 2b cells), they begin to express early neuronal markers such as Tbr2, which is specific to the development of glutamatergic excitatory neurons , along with DCX [3, 11, 20] and Igfbpl1 [20], marking their progression toward becoming immature neurons.

Neuroblasts (Type 3 cells), which are post-mitotic immature neurons, continue to express DCX along with Prox1, the latter being specific to excitatory granule cells in the DG. As neuroblasts further mature, they begin to express NeuroD1, a transcription factor crucial for the transition from neuroblasts to mature excitatory neurons [4, 21, 22]. As the neurons mature further, they begin to express markers characteristic of fully differentiated granule cells, including VGlut1/2 indicating their glutamatergic nature. NeuN, a general marker for mature neurons, is also expressed at this stage. Finally, fully mature granule cells express Calbindin, which distinguishes excitatory granule cells, while GABA is expressed in some interneurons in the DG [21, 23, 24].

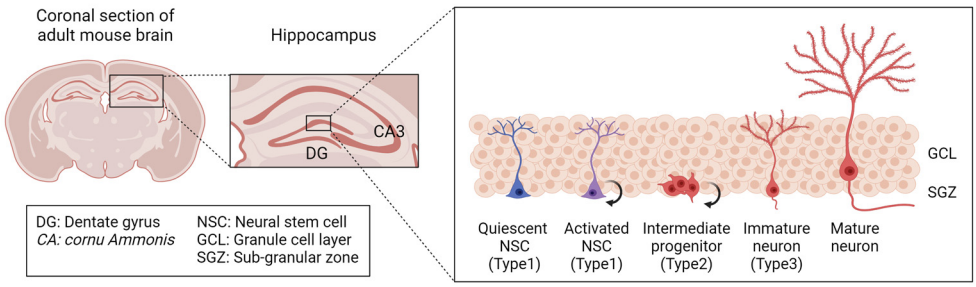

Figure 2 Schematic illustration of dentate gyral neurogenesis
In the sub-granular zone (SGZ) of the dentate gyrus (DG) lies radial glia-like, quiescent, NSC (Type 1) that get activated and differentiate past a proliferative intermediate progenitor state (Type 2) into immature neurons (Type 3, neuroblasts) that migrate a short distance towards the granule cell layer (GCL) where they mature into excitatory neurons. Created with BioRender.

New neurons that mature in the DG undergo a critical period of synaptogenesis, where they form connections and integrate with existing hippocampal circuits. The axons of these granule cells project to the CA3 region of the hippocampus, primarily forming synapses with pyramidal cells, while their dendrites extend into the molecular layer to receive inputs from the entorhinal cortex and amygdala, thereby affecting cognitive and emotional behaviors. This connectivity allows the newly formed neurons to contribute significantly to the overall plasticity of the hippocampal network, influencing processes like learning, memory formation, and emotional regulation through their involvemenet in stress response [23, 24].

The DG is a crucial region for memory encoding and spatial navigation. It acts as a gateway for information entering the hippocampus, contributing to the processing and encoding of memories by performing pattern separation, differentiating similar inputs into distinct outputs. This function is critical for distinguishing between similar experiences or environments, thereby preventing confusion in memory retrieval [25].

Subventricular zone neurogenesis

The subventricular zone (SVZ) lies along the walls of the lateral ventricles and is crucial for maintaining neural plasticity, particularly in the context of brain repair and olfactory processing, although its role in cognitive function is less prominent than that of the dentate gyrus. The SVZ houses a diverse population of neural stem and progenitor cells that contribute to neurogenesis throughout adulthood [8, 26, 27].

Type B cells, radial glia-like neural stem cells, reside in the SVZ, positioned adjacent to the ependymal layer lining the ventricles, with long processes extending into the brain

parenchyma. These cells express markers characteristic of neural stem cells, including GFAP, Nestin, and Sox2. As Type B cells proliferate and differentiate, they give rise to rapidly dividing transit-amplifying progenitors known as Type C cells. These intermediate progenitors express markers such as Ascl1 and Dlx2, both of which are important for their proliferation and differentiation. Ascl1 is a general marker of neural progenitors, while Dlx2 is more specific to the development of GABAergic (inhibitory) neurons, which are the predominant neuronal subtype generated in the SVZ [28-30].

As Type C cells mature, they give rise to Type A cells (neuroblasts) characterized by DCX and PSA-NCAM, which facilitate their migration along the RMS toward the OB. Upon reaching the OB, these neuroblasts differentiate into granule cells and periglomerular cells, which integrate into the olfactory circuitry and play roles in odor discrimination and sensory processing. Calbindin, primarily expressed in periglomerular cells, marks excitatory interneuron subtypes, while GABA, present in both granule cells and periglomerular cells, reflects their inhibitory function. These interneurons, once fully integrated, highlight the SVZ's essential role in sustaining olfactory function and neural plasticity throughout life [28, 29, 31, 32].

Olfactory bulb neurogenesis

Traditionally known to receive new neurons from SVZ via the rostral migratory sRMS, the OB also harbors its own population of resident NSCs. These stem cells, located mainly in the subependymal and glomerular layers of the OB, contribute to the ongoing neurogenesis of local interneurons, particularly granule cells and periglomerular neurons. Unlike the bulk of new neurons arriving from the SVZ, these OB-resident stem cells primarily support localized regeneration and neurogenesis, maintaining olfactory functions and responding to environmental cues, such as olfactory stimulation or injury [29, 32].

Research suggests that, under normal conditions, OB-resident progenitors produce inhibitory interneurons that contribute to the continuous turnover of neurons, allowing the OB to maintain its role in odor processing. Their activity increases under certain stimuli, such as enhanced olfactory demand or injury, highlighting their potential role in repair mechanisms independent of SVZ-derived neurons [29, 32].

Human neurogenesis

Anatomical differences between rodent and human brains lead to significant variations in neurogenesis. In humans, the SVZ is much smaller, with limited neuroblast migration to the olfactory bulb, while rodents maintain higher SVZ

activity throughout life. In addition, neurogenesis in the DG declines more rapidly with age in humans compared to rodents [33].

Human neurogenesis, remains a subject of intense study and debate due to its implications for cognitive functions and potential therapeutic interventions. Compared to mice, in which robust neurogenic activity is well-documented and critical for learning and memory, evidence for persistent neurogenesis in humans is weaker. Experiments involving postmortem brain samples, BrdU labeling, and carbon-14 dating have strongly suggested the presence of neurogenesis in adult humans throughout life [34-37]. However, recent studies have yielded conflicting results [38]. Taken togeter, current consensus is that while neurogenic activity in humans is less pronounced and declines more significantly with age than in rodents, it still occurs [37]. The debate underscores the complexity of translating findings from animal models to humans and highlights the need for further research to resolve these differences and fully understand the functional relevance of neurogenesis in the human brain.

Despite less pronounced neurogenesis in humans, it may still have important roles, particularly in brain repair following injury or neurodegenerative diseases. Neurogenesis decrease with age, contributing to cognitive decline and increased susceptibility to neurodegenerative diseases. Factors such as stress and inflammation can negatively impact neurogenesis, while physical exercise and mental stimulation can promote it. Understanding neurogenesis offers potential therapeutic avenues for enhancing brain function and treating conditions such as depression, Alzheimer's disease, and other neurodegenerative disorders. By fostering a conducive environment for neurogenesis, it may be possible to mitigate some of the cognitive declines associated with aging and improve overall brain health [33, 35-37].

The local microenvironment of different neurogenic niches

Neurogenesis in different brain regions is shaped by its surrounding microenvironments. In the subventricular zone (SVZ), ependymal cells line the ventricle, playing a critical role in facilitating signal exchange between the neural stem cells and their environment. For instance, ependymal cells help transport signals between the cerebrospinal fluid (CSF) and neural progenitors, while the vascular niche supplies oxygen, nutrients, and growth factors essential for maintaining neurogenesis [8, 39]. Additionally, astrocytes and microglia in the SVZ modulate neurogenesis by clearing apoptotic cells, managing inflammation, and secreting signaling molecules such as Wnt, Notch, and Sonic Hedgehog (Shh) [9, 11]. For example, astrocytes in

this region are involved in the secretion of growth factors that help regulate stem cell proliferation and differentiation [9]. The SVZ's direct exposure to CSF makes it more vulnerable to systemic influences, including inflammation, which can negatively impact neurogenesis by inhibiting stem cell activity [39].

In the dentate gyrus (DG) of the hippocampus, astrocytes play a more central role, with their influence being modulated by their proximity to mature neurons. These mature neurons provide feedback to integrate new neurons into established circuits. For instance, one study showed that astroglia in the DG promote neurogenesis by inducing adult neural stem cells to differentiate into neurons [40]. In contrast to the SVZ, microglia in the DG respond to local neuronal activity, fine-tuning neurogenesis based on synaptic plasticity rather than systemic signals [40, 41]. Another study found that DG microglia regulate the survival of neuroblasts through apoptosis-coupled phagocytosis, illustrating the importance of local neuronal feedback [41].

Both regions rely on the extracellular matrix (ECM) for structural support and the migration of progenitor cells. Molecules such as laminin and fibronectin are key components of this matrix, supporting cell adhesion and providing a scaffold for cell movement [30, 42]. However, while the SVZ is more exposed to systemic factors via the CSF, the DG's regulation of neurogenesis is more tightly coupled to local neuronal cues and synaptic plasticity, leading to different responses in these two regions to factors such as aging, injury, or inflammation [39] [42]. For example, one study provided quantitative evidence showing that ECM interactions are essential for stem cell migration in the SVZ [30], whereas another highlighted the inhibitory role of ECM components like chondroitin sulfate proteoglycans in limiting regeneration after central nervous system injury [42].

These distinct microenvironments, tailored to their respective niches, highlight the complexity of neurogenesis regulation and underscore how systemic factors and local cues can uniquely influence brain plasticity and regeneration throughout life.

Microglia and immune regulation

Microglia, the resident immune cells of the central nervous system (CNS), are key modulators of the neural microenvironment. They originate from progenitor cells in the yolk sac during early embryogenesis, playing a crucial role in shaping brain development and responding to injury or disease throughout life. These progenitors enter the developing brain in two waves, each contributing to different aspects of CNS formation. The first wave, at embryonic day 9.5 (E9.5), seeds early brain regions and

establishes a foundational microglial network. These early-arriving microglia are critical for brain patterning, vascular development, axon guidance, and the clearance of apoptotic cells during early neural development. The second wave, at embryonic day 12.5 (E12.5), replenishes and expands the microglial population, ensuring their widespread distribution throughout the brain. This second wave is believed to play a larger role in later processes, such as synaptic pruning and the refinement of neural circuits as the CNS matures [43, 44].

While both waves contribute to the overall microglial pool, the exact functional differences between them are still being explored. However, cells from the second wave may have more region-specific roles as the brain's architecture becomes more defined. Together, these waves establish a functional microglial network that supports CNS homeostasis. Throughout life, microglia remain highly dynamic, capable of altering their morphology and function in response to changes in the brain environment. This plasticity allows microglia to engage in protective and pathological processes, such as inflammation, neurogenesis, and neurodegeneration, highlighting their critical role in both brain health and disease [45, 46].

Microglial activation

In their normal resting state microglia constantly survey their environment, maintaining CNS homeostasis by clearing debris, supporting synaptic pruning and regulating neurogenesis. This active surveillance allows microglia to respond rapidly to changes in the brain's microenvironment, such as acute injuries like stroke or traumatic brain injury. In these cases, microglia initially undergo normal activation to contain damage, clear debris, and promote tissue repair. This early response often resembles what was traditionally categorized as M1 activation (pro-inflammatory), followed by a later phase of M2-like activation (anti-inflammatory, pro repair). However, this binary M1/M2 model is now viewed as oversimplified, with microglia responding through a continuum of activation states that vary according to the injury and environmental context [47, 48].

In response to chronic stressors like neurodegenerative diseases, infections, or aging, microglia can adopt more sustained activation states. Rather than using the M1/M2 classification, these activation states are now seen as tailored to specific situations. For example, in Alzheimer's disease, microglia shift to a neuroprotective state known as disease-associated microglia (DAM), where they clear amyloid plaques. Similarly, in aging, microglia enter a chronic low-level activation state making them more reactive to environmental stressors and contributing to persistent inflammation [48-52].

While microglial activation after acute injury, like stroke, is part of the normal protective response, prolonged or dysregulated activation can become maladaptive. These prolonged states, such as DAM in neurodegeneration, highlight microglia's adaptability but also their potential to cause sustained inflammation and impaired recovery [53, 54].

Microglia in neurogenic niches

Within neurogenic niches, microglia have specialized phenotypes that influence neurogenesis. In the DG, microglia have a hyper-ramified morphology, characterized by extensive branching and surveillance of the local environment. They play a dual role by promoting neuronal differentiation and neuroblast survival by releasing neurotrophic factors such as BDNF, while also clearing apoptotic newborn neurons through phagocytosis. This fine-tuned balance allows microglia to both support and regulate neurogenesis, ensuring that only the healthiest neurons integrate into existing circuits [41, 45, 55-59].

In the SVZ, microglia exhibit a more activated morphology, with an enlarged soma and fewer processes, typical of phagocytic cells. Interestingly, despite their morphology, SVZ microglia often display a resting phenotype and are less actively involved in phagocytosis compared to their DG counterparts. Instead, they are thought to support neurogenesis indirectly by maintaining a balanced immune environment, potentially through the release of anti-inflammatory cytokines and growth factors that support the proliferation and differentiation of neural progenitors [46, 57, 58].

Inflammation and its impact on neurogenesis

Inflammation impacts neurogenesis in both the SVZ and the DG, though the effects are nuanced and vary depending on the inflammatory mediators involved and the neurogenic niche. Under normal conditions, inflammation is a controlled process that supports tissue repair and defense. Acute inflammation can promote neurogenesis, particularly following injury or during repair mechanisms. For example, CXCL12 is upregulated during inflammation and enhances NPC migration, especially in the SVZ, guiding neural progenitors to damaged areas. In this context, IL-6 can act synergistically with CXCL12 to promote neuroblast proliferation [60-66].

However, chronic inflammation, a hallmark of aging and neurodegenerative diseases, generally impairs neurogenesis. Pro-inflammatory cytokines such as IL-1β, TNF-α, and sustained IL-6 signaling suppress the proliferation and differentiation of NPCs in both the SVZ and DG. The SVZ, located near the CSF and more exposed to systemic

inflammatory mediators, is particularly vulnerable to systemic inflammation. Chronic exposure to IL-6, for example, has been shown to impair neurogenesis by creating a toxic environment for NPCs, hindering their survival and differentiation [60, 61, 67].

Microglial activation plays a central role in mediating inflammation's impact on neurogenesis. CX3CR1, a receptor expressed on microglia, is essential for maintaining healthy communication between neurons and microglia. In the DG, CX3CR1 supports synaptic pruning and neurogenesis, while its loss disrupts these processes, leading to cognitive deficits [68, 69]. Activated microglia in both regions release TNF-α and IL-1β, which further exacerbate neurogenic decline by impairing proliferation of NPCs [70, 71].

Iba1 is a marker used to identify microglia. While Iba1-positive microglia play a role in clearing debris and regulating inflammation, their chronic activation, often leads to suppression of neurogenesis [72-74]. In contrast, microglia with low expression of Iba1 have been suggested to protect neurogenesis specifically in the SVZ, highlighting how inflammation affects neurogenic regions differently [46].

Interestingly, CXCR5/CXCL13 signaling, typically associated with leukocyte migration [75], also plays a role in neurogenesis. In the adult zebrafish brain, *Cxcr5* has been shown to regulate the proliferative capacity of radial glial cells (RGCs) and promote neuronal differentiation during regeneration [76]. In contrast, knockout of *Cxcr5* in the adult murine DG resulted in decreased proliferation and an increased number of progenitors [75].

Thus, while acute inflammation can enhance neurogenesis by promoting NPC migration and proliferation, chronic inflammation generally inhibits these processes. Importantly, studies show that blocking inflammatory pathways can restore neurogenesis, suggesting a potential therapeutic approach for mitigating the detrimental effects of chronic inflammation on brain plasticity [77].

Markers of immune cells and key inflammatory mediators

The dual role of inflammation in promoting and inhibiting neurogenesis and repair underscores the need to distinguish CNS-resident microglia from infiltrating peripheral immune cells, as their contributions to brain inflammation can vary significantly. Transmembrain protein 119 (TMEM119) and purinergic receptor P2RY12 are specific to microglia [78, 79], while CX3CR1 is predominantly expressed on microglia, and Iba1 marks both microglia and peripheral macrophages [80]. Inflammatory mediators can have different impacts based on their origin. CXCR5 is primarily found on

peripheral lymphocytes, while CXCR4 is expressed on microglia and neuroblasts, with the potential to directly influence neurogenesis [81, 82]. IL-6 is produced both in the CNS and peripherally [83], whereas USP18 and Lgals9 are predominantly peripheral but can cross the BBB and affect brain function [84, 85]. Understanding the distribution and function of these molecules is crucial in studying their role in brain aging and neurogenesis.

Cx3cr1

C-X3-C Motif Chemokine Receptor 1 (CX3CR1) is a chemokine receptor primarily expressed on microglia in the CNS and plays a crucial role in mediating microglia-neuron communication. Its ligand, CX3CL1 (fractalkine), is primarily expressed on neurons, creating a direct signaling axis that modulates microglial activity and influences several processes including synaptic plasticity, immune response, and neurogenesis [86] [87].

In the hippocampus, CX3CR1 has been shown to support the differentiation of NSCs into neuroblasts, aiding in the overall process of neurogenesis. Microglia expressing CX3CR1 regulate synaptic pruning and the maintenance of synaptic integrity, which is essential for cognitive function, particularly in learning and memory tasks. Studies indicate that a lack of CX3CR1 can impair neurogenesis, resulting in cognitive deficits, such as impaired learning and memory [68, 69]. However, the role of CX3CR1 in SVZ neurogenesis, particularly during aging, remains less understood, highlighting the need for further research.

Iba1

Iba1 (Ionized Calcium-Binding Adaptor Molecule 1) is a protein used as a marker for microglia in the brain and macrophages in peripheral tissues. It is involved in calcium binding and plays a key role in regulating actin cytoskeleton remodeling, which is crucial for microglial motility and phagocytosis. In the brain, Iba1 is expressed by both resting and activated microglia but is upregulated in response to injury and inflammation, facilitating the clearance of cellular debris and pathogens. Beyond the CNS, Iba1 is also expressed in macrophages in tissues such as the liver and bone, where it similarly supports immune and inflammatory responses [88, 89].

Cxcr5 / Cxcl13

C-X-C Chemokine Receptor 5 is a receptor that belongs to the family of G protein-coupled receptors (GPCRs) and is involved in the immune response. CXCR5 is primarily known for its role in guiding the migration of B cells and some T cells to specific areas within secondary lymphoid organs, such as lymph nodes and the spleen, through its interaction with its only known ligand, CXCL13. In the brain, microglia and neurons can express *Cxcl13* [90] and levels of CXCL13 in CSF increase in some neurological disorders such as Multiple sclerosis (MS) [91]. Interestingly, *Cxcr5* has also been suggested to regulate neurogenesis in adult zebra fish [76], and in the murine DG [75].

Cxcr4 / Cxcl12

CXCR4 is a chemokine receptor that interacts with its ligand CXCL12 (also known as stromal cell-derived factor 1, SDF-1) to regulate the migration of various cell types, including immune cells and stem cells. This signaling axis plays a crucial role in inflammation, as CXCR4/CXCL12 recruits immune cells to sites of injury or infection and regulates their migration through tissues. Additionally, CXCR4/CXCL12 is essential in stem cell homing, directing the movement of hematopoietic stem cells (HSCs) and other progenitors to specific niches in bone marrow and the brain. In the context of inflammation, CXCL12 is often upregulated, promoting immune cell migration, but persistent activation of this pathway can contribute to chronic inflammation and tissue damage [92-94].

Il6

Interleukin-6 (IL-6) is a cytokine that plays a dual role in the body. It is involved in the immune response by promoting inflammation during infections and tissue injuries, yet it also has regenerative functions. In the brain, IL-6 influences neurogenesis, and while it can support neural repair and growth, chronic overproduction is linked to neuroinflammation, which may impair neurogenesis and contribute to neurodegenerative conditions [83, 95].

Usp18

Ubiquitin-Specific Peptidase 18 (USP18) is a crucial regulator of the type I interferon (IFN) signaling pathway, acting as a negative feedback mechanism to limit immune activation. By modulating the JAK/STAT pathway, USP18 helps prevent prolonged

inflammation, stabilizing the ISG15 protein, which plays a role in antiviral defense and immune regulation. Beyond its role in controlling viral responses, USP18's activity is important for preventing excessive inflammatory responses that can lead to tissue damage. Dysregulation of USP18 has been linked to chronic inflammatory diseases, where its impaired regulation can exacerbate immune-related pathology. [84, 96].

Lgals9

Galectin-9 (Lgals9) is a β-galactoside-binding protein that plays a significant role in modulating immune responses and inflammation. It is known to promote immune tolerance by inducing the apoptosis of T-cells and regulating T-cell exhaustion, especially in chronic inflammatory conditions. Lgals9 also interacts with Tim-3, a receptor on T-cells and macrophages, helping to suppress excessive immune activation and promoting anti-inflammatory responses. Dysregulation of Lgals9 has been associated with autoimmune diseases, where its altered expression contributes to chronic inflammation [85, 97].

Aging

Aging is the gradual decline in biological function and resilience that unfolds over time. Unlike chronological age, which is a simple measure of time, biological aging can start at different times for different systems, often beginning in early adulthood. Cellular aging precedes any visible signs or functional decline, with the most pronounced effects typically emerging in middle age and later. However, despite recent progress, the mechanisms that initiate and drive the aging process remain largely unexplored [98].

Hallmarks of aging

The key biological processes contributing to aging have been organized into a framework called the hallmakrs of aging and are divided into three main groups based on how they contribute to and interact with the aging process.

Primary hallmarks are believed to be the initial triggers of cellular damage and dysfunction and consist of telomere attrition, genomic instabillity, epigenetic alternations, loss of proteostasis and disabled macroautophagy. Antagonistic hallmarks are responses to damage that can have beneficial or harmful effects depending on the context and intensity and consist of cellular senescence, mitochondrial dysfunction and deregulaed nutrient sensing. Integrative hallmarks are thought to be the end results of

damage and response mechanisms that lead to functional decline and aging and consist of dysbiosis, altered intercellular communication, stem cell exhaustion and chronic inflammation [98].

All of these hallmarks are interconnected, with inflammation and neurogenic decline being central themes that exacerbate the decline in brain function and contribute to age-related neurodegenerative diseases.

Age related disease

As we age, we become increasingly vulnerable to a range of common disorders, many of which are interconnected through inflammation, neurodegeneration, and cognitive decline. The four most common age-related brain disorders are Alzheimer's disease, Parkinson's disease, vascular dementia, and mild cognitive impairment. Alzheimer's disease and vascular dementia are considered dementia diseases, marked by progressive cognitive decline. Parkinson's disease, while primarily affecting movement, can also lead to cognitive issues in later stages. Mild cognitive impairment is a precursor to more severe dementia. All these disorders show varying degrees of disruption in neurogenesis [99].

Age related chronic inflammation

Age-related chronic inflammation, often referred to as "inflammaging," plays a pivotal role in the progression of brain aging and neurodegenerative diseases. This low-grade, persistent inflammation disrupts neurogenic niches [100], impairing both neurogenesis and cognitive function. A key driver of this chronic neuroinflammation is the release of inflammatory cytokines like IL-1β, TNF-α, and IL-6, which shift microglia from their homeostatic role into a chronically activated state. This shift diminishes the brain's ability to repair and regenerate, leading to cognitive decline commonly associated with aging [101].

As the brain's primary immune cells, microglia become increasingly prone to hyperactivation with age, amplifying inflammation and causing neuronal damage. Astrocytes, which normally support neurons and regulate the blood-brain barrier (BBB), also undergo significant changes during aging. They become reactive, releasing higher levels of pro-inflammatory cytokines, which not only activate microglia but also weaken the BBB. This astrocyte-driven inflammation exacerbates the cycle of neuroinflammation, creating a feedback loop between reactive astrocytes and microglia that contributes to further neuronal damage and accelerates cognitive decline in aging brains [102].

In addition to astrocytes, oligodendrocytes, responsible for myelination, also deteriorate with age. Oligodendrocyte dysfunction in aging leads to white matter damage, and this deterioration triggers further microglial activation. Activated microglia, in turn, release pro-inflammatory molecules that worsen oligodendrocyte damage, creating another layer of inflammatory feedback. This loss of myelin and the associated inflammation significantly contribute to the cognitive decline seen in aging, as communication between neurons becomes disrupted and neuroinflammation escalates [103].

Aging also compromises the BBB, which normally protects the brain from peripheral immune activity. With age, the BBB, supported by astrocytes, endothelial cells, and pericytes, becomes increasingly leaky. Dysfunctional pericytes contribute to this breakdown, allowing not only peripheral immune cells like T cells but also systemic inflammatory mediators, such as cytokines, to infiltrate the brain. These infiltrating factors add to the inflammatory burden, as T cells release interferon-gamma (IFN-γ), which suppresses neurogenesis and drives microglia into a prolonged activated state. This infiltration, combined with microglial activation is a major factor in the sustained neuroinflammation seen in aging [102].

At the borders of the brain, border-associated macrophages (BAMs) also contribute to the inflammatory processes. Positioned in regions such as the meninges and choroid plexus, BAMs respond to systemic inflammation, which increases with age, by releasing cytokines that influence brain inflammation. While BAMs do not penetrate the brain tissue itself, their activation enhances microglial responses, amplifying the inflammatory environment. This cross-talk between BAMs and microglia, along with systemic inflammatory mediators, particularly in the aging brain, further disrupts neurogenesis and accelerates cognitive decline, cementing the role of systemic and brain-resident immune cells in age-related neuroinflammation [104, 105].

Altogether, the aging process amplifies interactions between brain-resident cells, a leaky BBB, and infiltrating immune cells and mediators. This interplay fuels a cycle of chronic inflammation, neurodegeneration, and impaired regeneration, accelerating cognitive decline. Together, these mechanisms highlight the complex dynamics driving brain aging and its reduced capacity for repair [106].

Age related decline in neurogenesis

Neurogenesis persists throughout life, but declines significantly with age, contributing to cognitive impairments and reduced brain plasticity. All stages of neurogenesis are affected proliferation, differentiation, migration, and survival of neural progenitor cells, limiting the brain's regenerative ability as fewer neurons integrate into functional

circuits.This decline is influenced by both intrinsic factors within NSPCs and extrinsic, systemic changes, many of which are linked to age-related inflammation.

In the aging brain, pro-inflammatory cytokines strongly suppress neurogenesis. Chronically activated microglia release IL-1β, TNF-α, and reactive oxygen species, while reactive astrocytes secrete IL-6 and other pro-inflammatory molecules that inhibit NSPC activity. Elevated IL-1β reduces NSPC proliferation in both the SVZ and DG, while also impairing differentiation and survival of new neurons, particularly in the hippocampus, leading to reduced neuroplasticity and memory deficits. Similarly, TNF-α promotes apoptosis in the DG and disrupts the survival of newly formed neurons in both regions. IL-6 favors gliogenesis over neurogenesis, especially in the DG, where new excitatory neurons are vital for learning and memory. A weakened BBB accelerates the age-related decline by allowing increased BMP4 to enter the brain, inhibiting NSPC proliferation in the SVZ and particularly affecting the production of inhibitory neurons

In rodents, DG neurogenesis declines more rapidly than in the SVZ, affecting memory and learning functions early in aging. In humans, the opposite pattern is observed, with SVZ neurogenesis showing a sharp reduction early in adulthood, while DG neurogenesis, though reduced, continues at low levels into later life.

While neurogenesis is clearly affected by age-related inflammation, the complexity of these mechanisms and the regional differences between SVZ and DG requires further exploration of how age-related inflammation affect neurogenesis. This will be crucial for developing strategies to maintain neurogenesis and cognitive function during aging [107] [108].

Therapeutic Implications

The age-related decline in neurogenesis has profound implications for cognitive function. The hippocampus, a critical brain region for learning and memory, relies on the continuous generation of new neurons to maintain its plasticity and function. As neurogenesis declines with age, the hippocampus becomes less capable of forming new memories and adapting to new information, leading to cognitive impairments [109].

Stress significantly affects neurogenesis, especially in aging. Chronic stress elevates glucocorticoids like cortisol, which reduce neural stem cell proliferation and impair the survival of new neurons, particularly in the hippocampus. This accelerates age-related cognitive decline by increasing inflammation and oxidative stress, and can lead to hippocampal shrinkage and a higher risk of neurodegenerative diseases [110].

Furthermore, reduced neurogenesis is associated not only with normal aging but also with an increased susceptibility to neurodegenerative diseases like Alzheimer's. In Alzheimer's disease, impaired neurogenesis is strongly linked to the accumulation of amyloid-β plaques and tau tangles, which are key pathological markers driving the disease's progression [111]. This relationship suggests that enhancing neurogenesis in the aging brain could be a potential strategy for delaying or preventing the onset of neurodegenerative conditions.

Therapeutic strategies to counteract the decline in neurogenesis focus on modulating key signaling pathways, such as Wnt/β-catenin and brain-derived neurotrophic factor (BDNF), and targeting systemic factors to stimulate the production of new neurons. [112]. Additionally, lifestyle interventions such as physical exercise, cognitive stimulation, and dietary modifications have been shown to have beneficial effects on neurogenesis in the aging brain, offering non-pharmacological approaches to mitigating age-related cognitive decline [111].

The age-related decline in neurogenesis is a complex process driven by both intrinsic cellular mechanisms and extrinsic systemic factors. This decline has significant implications for cognitive aging and the development of neurodegenerative diseases. Understanding the molecular underpinnings of this process opens up avenues for therapeutic interventions aimed at preserving or restoring neurogenesis in the aging brain, potentially improving cognitive outcomes and quality of life for the elderly.

Aims of the thesis

This thesis aims to investigate the age-related decline in neurogenesis and its correlation with inflammatory changes both in the CNS and systemically.

Specific objectives

I. To determine if systemic prevalent CXCL13/CXCR5 signaling affects neurogenesis in the aged SVZ.

II. To investigate transcriptional changes in aged SVZ microglia and how the altered expression of Cx3cr1 impact neurogenesis during aging.

III. To explore how aging effect neurogenesis at the transcriptional level, particularly focusing on IPs.

Key results

We found that microglial, systemic and local immune changes, such as expression of microglia-predominant Cx3cr1, leukocyte-specific *Cxcr5*, systemic levels of cytokine Il6 and expression of chemokine Cxcl12 within the SVZ, specifically affect SVZ intermediate progenitors during aging. In addition, during normal aging, we identified a distinct subset of SVZ neuroblasts that develops an immune-related transcriptional profile, potentially playing a key role in how immune alterations influence neurogenesis.

Paper I. Loss of *Cxcr5* alters neuroblast proliferation and migration in the aged brain

As we age, inflammation changes both systemically and in the brain. These changes contributes to disruption of the blood-brain barrier (BBB) which allows greater infiltration of circulating cytokines [113]. Leukocytes in both blood and cerebrospinal fluid (CSF) express the C-X-C chemokine receptor 5 (CXCR5), which guides their migration towards its only known ligand, CXCL13 [114]. In this study, we investigated whether the loss of *Cxcr5* affects SVZ neurogenesis and the involvement of local and systemic inflammatory mediators.

Neuroblast numbers decreases in the SVZ and increases in the RMS of aged Cxcr5-/- mice

We found that Cxcl13 expression was higher in the aged brain, and hypothesized that loss of *Cxcr5* would affect neurogenesis during aging. To test this, we quantified proliferation and the number of different neurogenic cells in aged WT and *Cxcr5*$^{-/-}$ mice.

We used BrdU labeling in combination with cell type specific markers to show increased proliferation, particularly in neuroblasts, within the SVZ of aged *Cxcr5*$^{-/-}$ mice. Surprisingly, despite increased proliferation (Figure 3A, C), we detected a

decreased number of neuroblasts in the SVZ (Figure 3B) and increased number of neuroblasts in the RMS (Figure 3D) of aged *Cxcr5*$^{-/-}$ mice as compared to aged WT controls. Neuroblast proliferation were also higher in the RMS while newly formed neurons in the OB remained unchanged.

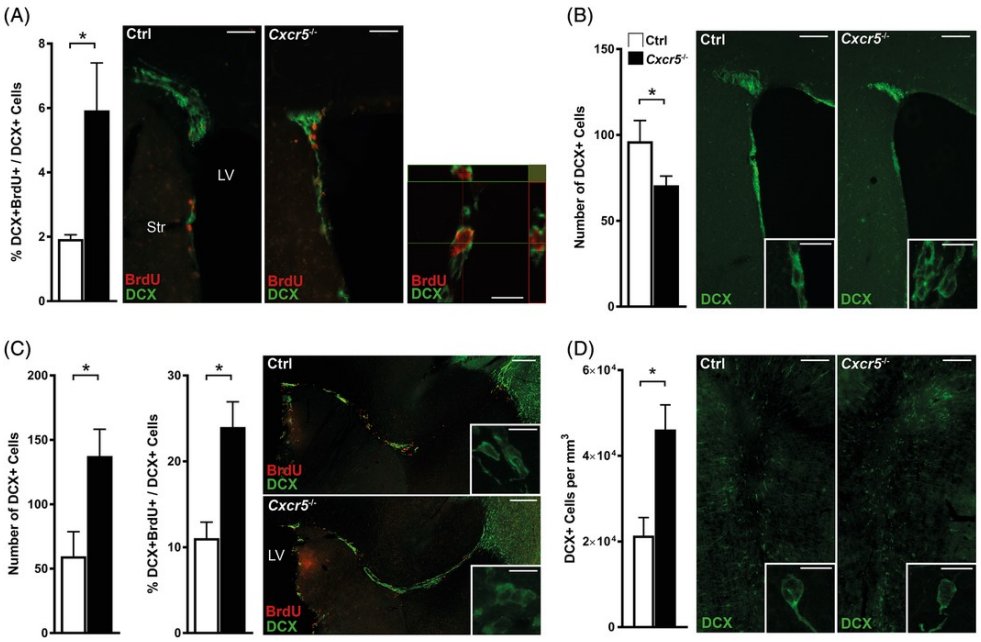

Figure 3 Loss of *Cxcr5* increase neuroblast proliferation and migration

(A) Quantification (left) and representative images (middle) of DCX+/BrdU+ cells in the SVZ of 18-month-old WT and *Cxcr5*–/– mice, along with an orthogonal projection confirming BrdU colocalization with DCX (right). (B) Quantification and representative images (overview and high magnification) showing the number of DCX+ cells in the SVZ of WT and *Cxcr5*–/– mice. (C) Quantification and example images (overview and high magnification) 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 images (overview and high magnification) showing the number of DCX+ cells in the OB of 18-month-old WT and *Cxcr5*–/– mice.

Data are presented as the mean percentage per 30 µm sections (A); as the mean number per 30 µm sections (B, C); and as the mean cell density (cells/mm³) per 20 µm section (D) ± SEM. Sample sizes are n = 5 (A, B), *Cxcr5*–/– n = 5, WT n = 4 (C), n = 4 (D). Statistical significance: *P < .05, determined with a two-tailed unpaired t test. Scale bars: 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.

Altered cytokine expression in aged Cxcr5-/- mice is a potential explanation for the changes observed in SVZ born neuroblast

To explore potential mechanisms for the altered neuroblast proliferation and migration, we quantified the expression of *Cxcr5* in FACS sorted neuroblasts from the SVZ of Dcx-GFP mice using QPCR, and analyzed previously published single cell RNA sequencing data from SVZ tissue. Surprisingly, Cxcr5 and *Cxcl13* expression was undetectable in all cell types, suggesting that the impact on neuroblasts is indirect, such as through changes in the microenvironment, alterations in cytokine signaling, or modulation of immune cell activity in the neurogenic niche.

Given the role of CXCR5 in immune cells and the fact that inflammation is a powerful regulator of neurogenesis [57], we investigated whether inflammation was altered in aged *Cxcr5*-/- mice. We did not observe any differences in microglial activation or immune signaling in the SVZ; however, we detected a significant increase in the inflammatory mediator IL-6 in the plasma of aged *Cxcr5*-/- mice (Figure 4A). Intrestingly, systemically delivered IL-6 have been suggested to have direct affect on SVZ neurogenesis [115] and to induce CXCL12 mediated chemotaxis and proliferation by upregulating *Cxcr4* [116]. Thus, we explored whether the absence of *Cxcr5* could affect the Cxcr4/Cxcl12 pathway, potentially explaining the observed effects on neuroblasts. While QPCR analysis of SVZ tissue showed no differences in *Cxcr4* expression, we observed increased expression of *Cxcl12* in the SVZ of aged *Cxcr5*-/- mice compared to controls (Figure 4E).

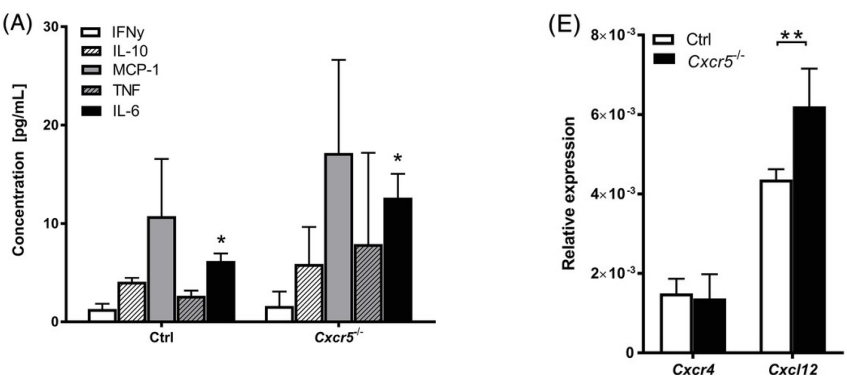

Figure 4 Loss of *Cxcr5* leads to increased levels of circulating IL-6, and *Cxcl12* expression in SVZ.
(A) Cytometric bead array analysis of circulating cytokine concentrations in plasma from 20-month-old WT and *Cxcr5*–/– mice, presented as mean concentration (pg/mL plasma). (E) mRNA expression levels of *Cxcr4* and *Cxcl12* in SVZ tissue from 20- to 22-month-old WT and *Cxcr5*–/– mice, assessed by RT-qPCR and normalized to Gapdh. Data are presented as mean fold change. Error bars represent ± SEM, with sample sizes of n = 4 for (A) and n = 3 for (E). Statistical significance: *P < .05, **P < 0.01, determined by two-tailed unpaired t test. SVZ, subventricular zone.

Taken together, our findings suggest that loss of *Cxcr5* increases proliferation and alters migration of SVZ born neuroblasts leading to lower number in the SVZ but increased numbers in the RMS, potentially mediated by increased levels of systemic IL-6 and *Cxcl12* expression in the SVZ.

Paper II. Microglia undergo disease-associated transcriptional activation and CX3C motif chemokine receptor 1 expression regulates neurogenesis in the aged brain

Having shown that *Cxcr5* deficiency during aging increases systemic and local inflammatory mediators targeting SVZ neuroblasts, we further investigated the relationship between aged microglia and neurogenesis. Microglia react to external signals [49] [52], which in Alzheimer's disease models induce an alternative activation state termed disease-associated microglia (DAM) [53, 54]. Similarly, during aging, SVZ microglia undergo chronic activation during [72], though its effects on SVZ neurogenesis remain insufficiently explored. Here, we investigate this connection to provide insights on how age-related microglial changes influence neurogenesis.

Aged microglia in the SVZ adopt a distinct DAM-like neuroprotective activation profile.

To explore age-related, niche-specific microglial changes that could impact neurogenesis, we analyzed previously processed RNA-seq data from adult and aged SVZ cells [117], which revealed two distinct microglial states (Figure 5A). One state was primarily microglia from young mice, the other predominantly from aged, referred to here as young- and aged-like SVZ microglia (Figure 5B-C).

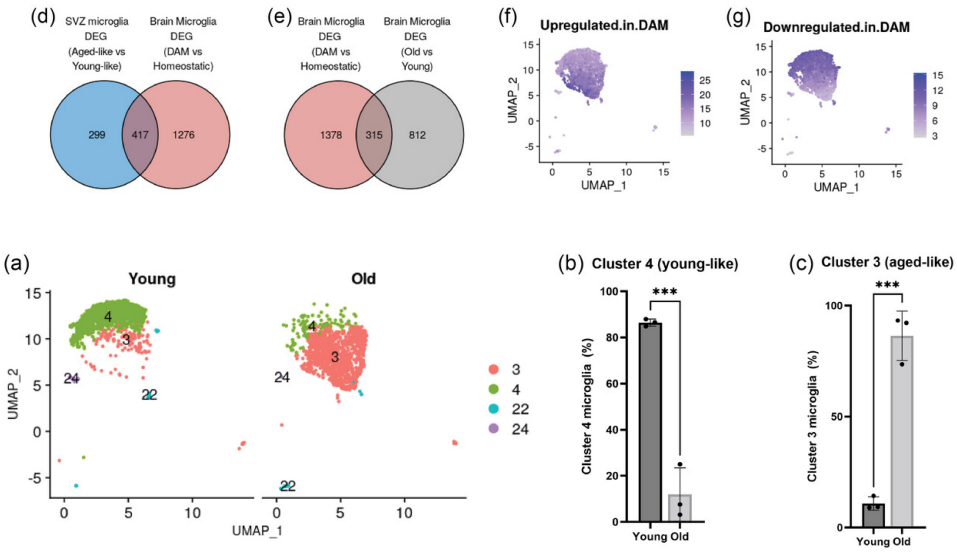

Figure 5. Microglia in the aged murine subventricular zone (SVZ) acquire a unique neuroprotective expression profile.
(A) UMAP plot showing the distribution of SVZ microglial states between young (2–3 months) and old (28–29 months) mice. (B) Quantification of Cluster 4 (young-like) microglia in the SVZ from young and old mice, expressed as cells per total microglia. (C) Quantification of Cluster 3 (aged-like) microglia in young and old mice, also expressed as cells per total microglia. (D) Venn diagram showing overlapping and unique differentially expressed genes (DEGs) between SVZ microglia (aged-like vs. young-like) and brain microglia (disease-associated microglia [DAM] vs. homeostatic microglia). (E) Venn diagram showing overlapping and unique DEGs between brain microglia from young (2–3 months) and old (21–22 months) mice and brain microglia (DAM vs. homeostatic). (F) UMAP plot of SVZ microglia showing combined expression of genes upregulated in DAM. (G) UMAP plot of SVZ microglia showing combined expression of genes downregulated in DAM. Data are presented as mean values, n = 3 for (B) and (C), demonstrating how aged microglia exhibit unique transcriptional changes and share certain characteristics with DAM in the aged brain.

We compared DEGs previously identified in DAM from the whole brain [118] with those we found between different age groups of microglia in the SVZ and whole brain [119]. Interestingly, aged-like SVZ microglia shared a majority of DEGs with DAM (Figure 5D, F-G), whereas aged microglia from the whole brain only shared a few (Figure 5D). We discovered that SVZ microglia exhibit a unique aging pattern by comparing DEGs between young and old microglia from SVZ and the whole brain. In addition, several genes upregulated in aged-like SVZ microglia were linked to negative regulation of neuronal death and extrinsic apoptotic signaling, suggesting a neuroprotective role.

CX3CR1 heterozygosity enhances neurogenesis in the aged SVZ

CX3CR1 is not only a highly specific microglia marker in the murine brain [86], but has also been suggested to support hippocampal neurogenesis [68, 69]. Interestingly,

we observed a decrease in *Cx3cr1* mRNA expression specifically in aged-like SVZ microglia which coud have a potential affect on neurogenesis.

To investigate the impact of reduced CX3CR1 levels on SVZ neurogenesis, we compared neuroblast density and proliferation in the SVZ between WT and *Cx3cr1*+/- mice. Using immunohistochemistry, we labeled neuroblasts with the marker DCX and proliferating cells with MCM2. Our analysis revealed a significant increase in proliferation and density of neuroblasts in the aged *Cx3cr1*+/- SVZ compared to aged WT (Figure 6F, H) but not between adult counterparts (Figure 6C, E). Notably, we found a reduction in the number of neuroblasts in the DG of aged *Cx3cr1*+/- mice, while proliferation remained unchanged.

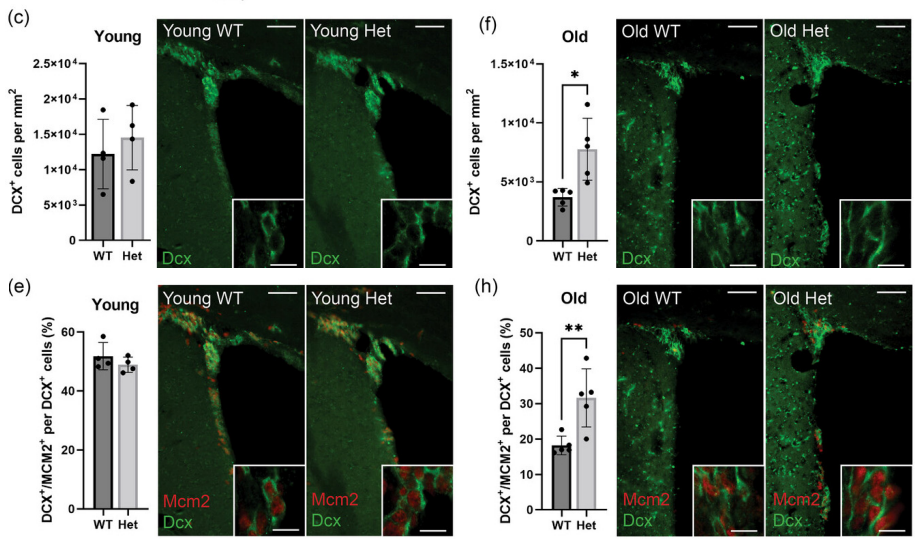

Figure 6. Decreased expression of *Cx3cr1* leads to increased proliferation and neuroblast density in the aged subventricular zone (SVZ).
(C) Violin plot illustrating the expression of *Cx3cr1* in Cluster 4 (young-like) and Cluster 3 (aged-like) SVZ microglia. (E) UMAP plot showing the distribution of *Cx3cr1* expression in SVZ microglia. (F) Quantification and representative images (overview and high magnification) of doublecortin-positive (DCX+) cells in the SVZ of young (3–4 months old) WT and Cx3cr1+/– mice. (H) Quantification and representative images (overview and high magnification) of DCX+ and MCM2+/DCX+ cells in the SVZ of old (17–19 months old) WT and *Cx3cr1*+/– mice. Data are presented as mean cell density (cells/mm²) per 30 µm section ± SEM; n = 4 (C, F), n = 5 (E, H). Scale bars: 50 µm (overview), 10 µm (high magnification).

In summary, aged SVZ microglia display a unique DAM-like neuroprotective gene expression profile with reduced *Cx3cr1* expression, and *Cx3cr1* heterozygosity increases neuroblast proliferation and density in the aged SVZ but not in the DG.

Paper III. Single cell RNA sequencing of aging neural progenitors reveals loss of excitatory neuron potential and a population with transcriptional immune response

Having shown that local and systemic mediators, along with microglial changes, specifically influence neuroblasts during aging, we sought to further investigate these IPs in the aging process. To enrich for, and study the transcriptional effects of aging on IPs, we dissected the SVZ, OB, and DG from 3-, 14-, and 24-month-old DCX-GFP mice, isolated GFP+ cells with FACS, and performed bulk and single-cell RNA sequencing.

Aged neuroblasts display subtle and dynamic transcriptional changes

We performed unbiased clustering of cells from all ages and regions, pooled digitally, and annotated clusters using published RNA seq data and known markers. Most IPs differed between the DG and the SVZ, while the OB contained all stages of IPs found in the SVZ including a unique neural stem cell like population.

Differential gene expression analysis on clusters from neuronal lineages between adult and aged cells in the SVZ, OB, and DG revealed few up- or downregulated genes in most aged cell types. In the SVZ, no DEGs were common across all cell types, and while no diffrential gene signature were the same, closer neighboring cell states shared more DEGs. Interestingly, several genes had an expression maximum in the mid aged group that droped or went back to normal levels in the aged, suggesting that transcriptional changes during aging are not necessarily monotonic (Figure 7).

Figure 7. Non monotonic gene expression differences during aging across neurogenic cell tyes in the subventricular zone (SVZ).
Extreme point plot highlighting genes with maximum or minimum expression in the middle-aged group across different SVZ clusters. Colored dots indicate genes with significant differential expression compared to the adult stage, emphasizing non-monotonic patterns of gene expression during aging.

Excitatory neuron progenitors in SVZ- and DG decrease faster during aging than inhibitory neuron progenitors

After showing that most IPs undergo only minor age-related changes, we focused on populations that changed in size with age (Figure 8B). NSCs in the adult DG primarily generate excitatory neurons, while SVZ neurogenesis produces inhibitory interneurons [3]. To study IP heterogeneity during aging, we identified progenitors destined for different neuronal subtypes using established marker genes. Gad1 (Gad67) and St18 marked IPs for inhibitory interneurons, and Eomes (Tbr2), Tbr1 and Slc17a6 (Vglut2) marked IPs for excitatory neurons. We found that a fraction of SVZ IPs clustered together with DG IPs and expressed markers for excitatory neurons.

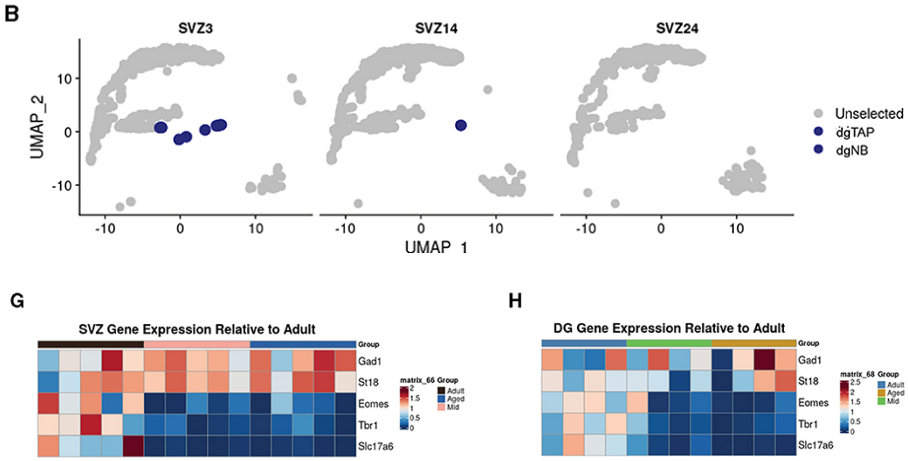

Figure 8. Excitatory neuron progenitors in the SVZ and DG decrease faster during aging than inhibitory neuron progenitors.

(B) Cluster dgTAP and dgNB highlighted in different age groups of the SVZ, illustrating the decline in excitatory progenitors with aging. (G, H) Heatmaps showing gene expression of common markers for inhibitory neurons (Gad1 and St18) and excitatory neurons (Eomes, Tbr1, and Slc17a6) in bulk samples, relative to mean adult expression levels, for the SVZ (G) and DG (H), further emphasizing the differential impact of aging on excitatory versus inhibitory progenitors.

Our single-cell data revealed that Eomes (Tbr2)-positive IPs in both neurogenic regions declined more rapidly with age compared to Gad1-positive Ips (Figure 8G). To confirm this, we performed statistical analysis on bulk RNA-seq data, which showed a faster age-related decrease in the expression of Eomes (Tbr2), Tbr1, and Slc17a6 (Vglut2) in the SVZ and DG, compared to Gad1 and St18 in the SVZ (Figure 8H). Together, this suggests that IPs destined to become excitatory neurons decline faster with age than those fated to become inhibitory interneurons.

A subset of aged SVZ neuroblasts develop an immune-related expression profile

We observed that most cells in our dataset had minimal or no age-related inflammatory changes. Therefore, we explored cluster NB4, which was scarcely present in adult samples but expanded with age in the SVZ (Figure 9A-B), while remaining absent in the OB and DG across all ages.

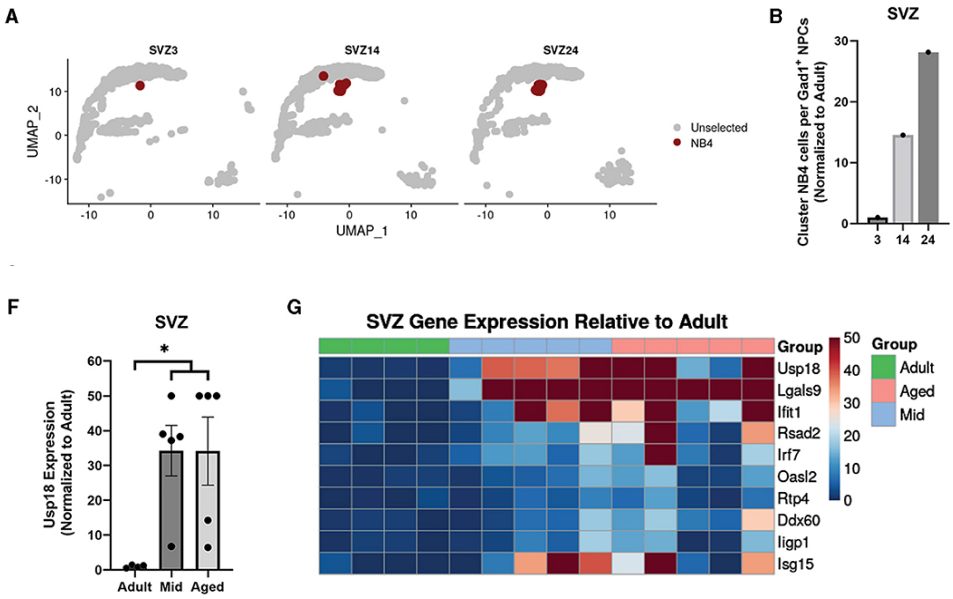

Figure 9. A distinct population of neuroblasts in the aged SVZ acquires an immune-related expression profile. (A) UMAP plot highlighting cluster NB4 across different age groups in the SVZ, showing its increased presence with aging. (B) Quantification of cells in cluster NB4 compared to non-cycling populations expressing Gad1 in the SVZ, represented as a ratio, illustrating the distinct expansion of NB4 with age. (F) Gene expression of Usp18, normalized to adult levels, in bulk sequencing samples of the SVZ, with significant differences (*p.adj < 0.05*) between adult and combined middle-aged/aged samples. (G) Heatmap showing gene expression of the top defining genes of cluster NB4 from bulk samples, relative to the mean adult expression in the SVZ, emphasizing the immune-related profile of this cluster.

Interestingly, when compared to Gad1+ IPs, we found that NB4 exhibited a gene expression profile similar to neuroblasts, but with several genes upregulated in response to innate immune activity and external biotic stimuli. Notably, Usp18 was highly enriched, and Lgals9 was specific to NB4. Analysis of bulk RNA seq data of SVZ tissue further confirmed increased expression of these immune-related genes in mid-aged and aged samples compared to adults (Figure F-G), strongly suggesing that a subset of neuroblasts acquires an age-related immune response.

Taken together, our RNA expression data suggest that excitatory neuron-fated IPs decline more rapidly with age than inhibitory, regardless of neurogenic regions. In additioin, most IPs in aged neurogenic niches show minor immune-related gene expression changes, while a distinct subset in the SVZ acquires a pronounced immune response profile.

Discussion and future perspectives

We found that local, microglial, and systemic immune changes specifically affect SVZ intermediate progenitors during aging, with a distinct subset acquiring an immune-related transcriptional profile. Interestingly, DG neurogenesis responded differently to aging and to altered inflammatory signaling compared to the SVZ.

Inflammation drives age-related changes in neurogenesis that varies across niches.

The microenvironment differs considerably between neurogenic regions, with the SVZ positioned near the ventricles, in close contact with cerebrospinal fluid and ependymal cells [27, 120], making it more susceptible to systemic changes. In contrast, the DG is located closer to mature neurons influenced by local neural activity and synaptic inputs. [26]

Cxcr5 is primarily expressed on immune cells outside the brain, while Cx3cr1 is expressed on brain-resident microglia. We found that loss of Cxcr5 and Cx3cr1 heterozygosity increase proliferation in the aged SVZ seperately in paper I and II, respectively, while previous studies showed that Cx3cr1 and Cxcr5 deficiency seperately reduced proliferation in the adult and aged DG [68, 75]. The differences, together with our observation that SVZ neurogenesis doesn't change in adult Cxcr5-/- or Cx3cr1+/- mice, suggest that loss of Cxcr5 and Cx3cr1 heterozygsity has region- and age-specific effects on neurogenesis.

We did not detect any expression of Cxcr5 in sorted neuroblasts in paper I, suggesting the observed effects are indirect. Although, we saw no signs of altered microglia activation, plasma from aged Cxcr5-/- mice contained higher levels of IL-6. Systemic IL-6 can cross the BBB and reach the SVZ, where it acts directly on SVZ cells to promotes neuroblast proliferation [115, 121-123]. This suggests that elevated circulating IL-6 in aged Cxcr5-/- mice may partly explain the increased proliferation. The proximity of SVZ to blood vessels and CSF makes it more accessible to circulating cytokines compared to the DG [30, 120], potentially explaining the regional differences

observed, with increased proliferation in the aged SVZ but not in the DG. While numerous studies have examined the susceptibility of different regions to circulating cytokines [124, 125], a direct side-by-side comparison between regions, such as with IL-6, would be highly valuable not only in relation to aging.

While the SVZ is more susceptibe to systemic changes, crosstalk between microglia and neurons via CX3CR1/CX3CL1 signaling [126] is particularly prominent in the DG, where local neural activity plays a greater role compared to the SVZ. Our findings in paper II indicate that aged SVZ microglia develop a distinct expression profile compared to other brain regions. This, combined with the DG's proximity to neurons, may help explain the region- and age-specific effects of Cx3cr1 haploinsufficiency[26, 127].

Moreover, NSCs in the SVZ give rise to inhibitory interneurons for the olfactory bulb, while DG NSCs are more quiescent and have a restricted differentiation potential, primarily generating excitatory granule neurons [8, 26, 27, 128]. These intrinsic differences between neurogenic cells, and how they are differentially affected by aging, as we demonstrated in paper III and others have shown [129], may further contribute to region- and age-specific variations.

Intrinsic differences between neural stem cells (NSCs) in the SVZ and DG are shaped by their embryonic origins, with SVZ NSCs arising from the lateral ganglionic eminence (LGE) and DG NSCs originating from the medial ganglionic eminence (MGE) and cortical neuroepithelium [32]. While these developmental origins contribute to their distinct properties, studies show that when NSCs are transplanted into a different neurogenic niche, their multipotency, turnover rate, and lineage potential adapt to the local environment [130-132]. This suggests that extrinsic factors in the niche can override some of the intrinsic characteristics of NSCs. The local niche and anatomical differences, such as proximity to CSF and neurons, play a dominant role in regulating these properties, often superseding the intrinsic characteristics of NSCs in their native location. This likely serves as a key driver of region- and age-related variations.

Clinical interventions targeting inflammatory pathways include CXCR5/CXCL13 blockade, explored in autoimmune diseases like multiple sclerosis to reduce neuroinflammation and immune cell infiltration [133, 134]. Additionally, IL-6 inhibitors, such as tocilizumab, treat inflammatory conditions like rheumatoid arthritis and are under investigation for reducing neuroinflammation in neurodegenerative disorders [135]. Taken together, it is crucial to consider the distinct effects that clinical interventions targeting inflammatory pathways may have on neurogenic regions in patients of different ages.

Aged microglia are critical regulators of neurogenesis with dual effects

With aging, the brain undergoes chronic low-grade inflammation, marked by persistent microglial activation and elevated pro-inflammatory cytokines like IL-6 [136]. This inflammatory state have been shown to accelerate neurodegenerative diseases like Alzheimer's and disrupt neurogenesis. [137]

In paper II, we demonstrate that aged SVZ microglia undergo distinct transcriptional changes, closely resembling DAM and showing potential neuroprotective properties. The aged brain is reported to harbor 3% DAM [138], and age-related dysregulation may interfere with microglial transition to DAM [118, 139]. The unique transition observed in aged SVZ microglia could reflect priming toward full activation, premature arrest, or adaptation to cues distinct from Alzheimer's pathology. New region-specific aging datasets are crucial to better understand the distribution of microglia with different activation states, which can be captured through spatial transcriptomics methods.

Microglia, particularly DAM, clear extracellular protein aggregates and play neuroprotective roles in neurodegenerative diseases. In aged neurogenic niches, a subset of quiescent NSCs (qNSCs) exhibits fewer and defective lysosomes, leading to reduced autophagy and accumulation of protein aggregates, impairing their activation [140, 141]. Enhancing lysosomal function and autophagy can counter these effects [142]. It is possible that DAM in the aged SVZ support neurogenesis by clearing protein aggregates from NSCs, though whether they directly influence lysosomal function in qNSCs remains to be determined.

Apoptotic immature neurons in the hippocampus decline with age and are normally cleared by phagocytic microglia [41, 143]. We found that P2ry12, essential for phagocytosis [144], was specifically downregulated in aged-like SVZ microglia. Interestingly, reduced P2ry12 expression leads to decreased phagocytic activity, which have been linked to impaired neurogenesis [145]. While phagocytic activity declines in the aged brain, including the hippocampus [146], DAM show increased activity to clear protein aggregates providing neurprotecion [54]. Microglia also regulate apoptosis via their secretome, affecting the survival of newly formed neurons [147]. We identified several DEGs in aged-like SVZ microglia that could potentially reduce apoptosis and offer neuroprotection, though the extent of their protective role remain unclear.

Neuroprotection in aged-like SVZ micrglia is suggested by their similarities to DAM, but substantial evidence suggests that certain aspects of alternative activation

in aged microglia remain detrimental to neurogenesis [148, 149]. It is therefore crucial to further investigate which age-related microglial changes promote neurogenesis and which impair it, as well as their broader impact on other processes within the neurogenic niche.

While microglia have large impact on surrounding cells and neurogenesis, its also important to understand what drives age-related changnes in microglia as they aquire the most prominent age- related changes of all cells in the SVZ [150]. In paper I, we demonstrate that loss of Cxcr5 indirectly impacts neurogenesis, potentially through elevated systemic levels of IL-6. In paper II, we show that Cx3cr1 heterozygosity, downregulated in aged SVZ microglia, also influences neurogenesis. Although we observed no clear phenotypic or morphological changes in microglial activation due to Cxcr5 deficiency, a higher-resolution analysis, such as transcriptomics, could provide a more detailed picture. Regardless, this raises the possibility that other immune cells influencing neurogenesis may have been affected in both scenarios.

Recent studies show that border-associated macrophages regulate neurogenesis and drive neuroinflammation in age-related diseases where microglia have a less detrimental role [151] [152]. In addition, T-cell infiltration into the aged SVZ have the potential to impar neurogenesis [117, 153], highlighting the need for further research on immune cell interactions in aging neurogenic niches and their contribution to neurogenic decline.

Age-related inflammatory changes regulates neurogenesis and can act specifically on neuroblasts

We found that the loss of Cxcr5 and Cx3cr1 heterozygosity separately and specifically affects SVZ neuroblasts during aging, as shown in Papers I and II, respectively. In addition, we demonstrate in Paper II that during normal aging, a subset of neuroblasts in the SVZ acquires a transcriptional immune response, which was not observed in other regions or cell types

Prevous studies support our findings that neuroblasts are particularly sensitive to inflammation, more so than other cells in the neurogenic niche. They show that inflammatory cytokines like IL-1β, TNF-α, and IL-6 significantly reduce neuroblast proliferation, impair their migration by disrupting CXCL12/CXCR4 signaling, and increase their rates of apoptosis [57, 77, 154]. In contrast, NSCs and astrocytes exhibit greater resilience to inflammation, with NSCs able to remain quiescent or self-renew

[155], and astrocytes and microglia often becoming reactive [156] which mean they survive and adapt. This suggests that neuroblasts are uniquely vulnerable to inflammation, leading to more significant disruptions in neurogenesis during aging and neurodegenerative conditions. Therefore, targeting inflammatory processes may help preserve neuroblast function and support neurogenesis in these contexts.

Aged Cxcr5-/- mice had fewer neuroblasts in the SVZ, with accumulation in the RMS that didn't lead to more new neurons in the OB. In paper III, we identified all stages of SVZ intermediate progenitors within the OB, along with a unique population of neural stem cells, consistent with previous reports [157]. Since we didn't observe any increase in senescent cells in the Cxcr5-/- mice, it is possible that the production of new neurons in the OB is regulated by the increased influx of neuroblasts from the SVZ to maintain homeostasis. It would be valuable to investigate the interaction between SVZ and OB neurogenesis in greater detail by tracing progenitors from different origins while selectively blocking or inducing neurogenesis in one of the regions. This could be achieved through targeted injections of GFP-labeled retrovirus into the lateral ventricle and OB to trace progenitor migration [28], and EGF to stimulate neurogenesis [158].

Its is interesting to speculate that neuroblasts are involved in regulating neurogenesis in different regions. In our sequencing data in paper III, while most IPs showed minor changes with age, we identified a population of neuroblasts in the mid-aged and aged SVZ with a strong immune-responsive gene expression, marked by increased Lgals9. This population was absent in the OB, suggesting these neuroblasts may be inhibited from reaching their destination and maturing into neurons. This could either be a mechanism to select healthy neuroblasts or a result of an altered inflammatory environment. Either way, it might contribute to the reduction in new neurons in the OB. It would be interesting to see if this population is affected in Cxcr5 deficient mice and contributing to neuroblast migration or differentiation.

It is possible that the immune response in IPs may be triggered by increased T-cell infiltration into the aged SVZ [150, 159], with Lgals9 acting as a T-cell suppressor [160], but could also be the other way around. Further studies could investigate whether depleting T-cells prevents the appearance of this reactive neuroblast population during aging.

Our findings in Paper III show that IPs destined to become excitatory neurons decline more rapidly than those fated for inhibitory neurons, with DEGs in IPs not following a monotonic pattern [161] but instead peaking in mid-aged groups. This suggests that inflammatory changes, which arise as part of the aging process, may start affecting neuroblasts earlier than expected, potentially triggering pathways that lead to

detrimental outcomes later in life. Assessing additional intermediate time points is crucial, as these early shifts could shape the trajectory of aging, rather than being consequences that manifest only in advanced age. The specific vulnerability of neuroblasts to inflammation, coupled with the variation across neurogenic niches and the dual role of aged microglia, further influences these temporal dynamics. Ultimately, understanding the spatial and temporal aspects of how inflammation impacts neurogenesis is essential for unraveling the full trajectory of aging-related neurogenic decline.

Concluding remarks

In paper I, we show that germline loss of Cxcr5 specifically affects neuroblast proliferation and migration in the aged brain, likely via elevated IL-6 and CXCL12, highlighting the need for further research into the role of other immunomodulatory factors in age-related neurogenic decline. Clinically, this is significant as CXCR5/CXCL13 inhibition is proposed as a therapy for neuroinflammation [133]. Our findings suggest that long-term inhibition of this pathway could impair neurogenesis in older patients.

In paper II, we demonstrate that aged SVZ microglia acquire a disease-associated profile with decreased Cx3cr1 expression and identifies CX3CR1 as a novel age- and niche-specific regulator of adult neurogenesis. These findings suggest that SVZ microglia adjust gene expression to mitigate neurogenic decline in aging, emphasizing the need for further research on microglia-neural stem cell interactions over time.

In Paper III, we show that excitatory neuron-fated IPs decline faster with age than inhibitory IPs, regardless of region, and while most aged IPs show minor immune-related changes, a distinct subset in the SVZ develops a pronounced immune response. These findings highlight the potential importance of targeting excitatory neurogenesis to address age-related memory loss and suggest that pathways involving Usp18 and Lgals9 could be therapeutic targets for mitigating inflammation-related neurogenesis impairments.

Altogether, we found that local, microglial, and systemic immune changes specifically impact SVZ intermediate progenitors during aging, with a distinct subset acquiring an immune-related transcriptional profile. Notably, DG neurogenesis responded differently to aging and inflammatory signals compared to the SVZ. This highlights inflammation as a key driver of age-related changes in neurogenesis, capable of affecting specific cell populations differently across niches, with both positive and negative effects. These findings underscore the importance of considering niche- and age-specific responses when developing clinical interventions targeting immune cells or signaling pathways.

Key methods

Cell Culture and Neurosphere Assay

In a typical neurosphere assay, we isolate NSPCs from regions like the SVZ and culture them at clonal density in a controlled environment. We maintain these cells in a humidified incubator at 37°C with 5% CO2 to promote optimal growth. The standard media for expanding NSPCs includes DMEM/F12 supplemented with L-glutamine, B27, penicillin-streptomycin, and growth factors such as epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF). Fresh media with growth factors is added every two days to ensure optimal conditions. We typically quantify the formation of neurospheres by counting the number of spheres formed and measuring their diameters using imaging software, as these clusters of NSPCs represent their ability to self-renew and differentiate.

Discussion of Alternative Methods

There are several alternative methods for assessing the proliferation and differentiation potential of NSPCs. The neurosphere assay, while widely used, has limitations, such as being biased towards cells that grow well in suspension. Adherent monolayer cultures provide an alternative, where NSPCs are grown on coated plates, allowing for more precise control of cell density and differentiation. Additionally, 3D organoid cultures are gaining popularity as they offer a more physiologically relevant environment compared to traditional neurosphere assays. These newer methods can provide complementary insights, but the choice of method depends on the research focus—whether it is self-renewal, differentiation potential, or creating a more complex brain-like structure.

Cytometric bead array

In a Cytometric Bead Array (CBA), we simultaneously detect multiple cytokines in biological samples such as plasma, serum, or cell culture supernatants. We incubate a small sample volume with a mixture of beads, each coated with capture antibodies specific to different cytokines (e.g., IL-6). After incubation, we add a secondary detection antibody and analyze the sample using flow cytometry, where the beads pass through a laser, and the fluorescence intensity is measured for each cytokine. The resulting data is quantified and presented as concentrations.

Discussion of Alternative Methods

This method offers advantages over traditional ELISA, as it allows simultaneous measurement of multiple cytokines from a single sample, saving time and reducing sample consumption. In comparison, Luminex is another multiplex technique that uses bead-based technology with a different detection platform. Luminex employs color-coded beads and a specialized reader to measure multiple analytes in one sample, offering a broader capacity for high-throughput analysis, making it ideal for large-scale studies with many samples and analytes.

For studies with a smaller number of samples and a focus on precision, CBA is often preferred, as it integrates well with existing flow cytometry systems. However, for higher throughput and larger datasets, Luminex may be more suitable due to its ability to process numerous samples simultaneously with minimal hands-on time. The choice between the two methods depends on the scale of the experiment and available equipment.

Brain dissection and tissue dissociation

We perform brain dissection and tissue dissociation as standard techniques to isolate specific brain regions, such as the SVZ, for downstream applications like RNA isolation or cell culture. In a typical protocol, we sacrifice the animals and quickly collect the brains in ice-cold media, such as L-15, to preserve tissue viability. For RNA isolation, we either process whole brains or dissect specific regions like the SVZ, either immediately or by storing them at -80°C in lysis buffer for later analysis. For cell culture applications, we further process the dissected brain regions using tissue dissociation kits, which help break down the tissue into single cells. Once dissociated, we purify the cells

using density gradients or other purification methods, and use them in various assays, such as neurosphere formation or cell differentiation studies.

Discussion of Alternative Methods

Alternative methods for brain dissection and tissue dissociation depend on the goals of the experiment. For example, manual dissociation using enzymatic digestion followed by mechanical trituration is a more traditional approach, but modern dissociation kits provide more standardized and reproducible results. Laser capture microdissection (LCM) is another advanced technique that allows for the precise isolation of specific brain cell populations from tissue sections. While LCM offers high precision, it requires specialized equipment and expertise, making it more suited for targeted studies of small cell populations. The choice of method should be based on the specific needs of the research, such as whether the focus is on high-throughput cell isolation, RNA integrity, or the need for precise dissection of brain subregions.

Fluorescence-activated cell sorting

We use fluorescence-activated cell sorting (FACS) to isolate specific cell populations from heterogeneous mixtures based on the expression of fluorescent markers. For example, in studies focused on neural progenitor cells, we dissociate cells from brain tissue and sort them using FACS based on the expression of markers such as GFP (green fluorescent protein), which can be genetically introduced or expressed endogenously. We often include additional markers, such as propidium iodide, to assess cell viability, ensuring that only live cells are collected for further analysis. We typically use FACS machines, like the FACSAria series from BD Biosciences, to perform the sorting, and the sorted cells are collected into a suitable medium for downstream applications, such as single-cell RNA sequencing or bulk RNA sequencing, depending on the experimental goals.

Discussion of Alternative Methods

While FACS is highly effective for isolating specific cell types based on fluorescence, it can be labor-intensive and requires specialized equipment. Magnetic-activated cell sorting (MACS) offers an alternative for cell enrichment, using magnetic beads conjugated to antibodies that bind target cells. While MACS is faster and less costly than FACS, it lacks the precision of FACS in isolating single cells and may result in

lower purity. Another alternative is microfluidic-based sorting systems, which offer high-throughput and gentle cell handling, but these systems are still less widely available and generally more suited for specific applications like circulating tumor cells. The choice between these methods depends on factors such as the need for high purity, sample throughput, and the availability of specialized equipment.

Imaging and quantification

We typically use advanced microscopy for imaging and quantification in neuroscience research to analyze specific cell populations and their markers. For co-localization studies, we employ confocal microscopes, such as the Zeiss LSM 780 with high-magnification objectives, to visualize multiple markers within the same cell or tissue. For image acquisition and analysis, we use software tools like Zen for confocal microscopy. For broader cell quantifications, we often use epifluorescence microscopes like the Olympus BX61. Quantification of cells, including dividing cells or markers such as BrdU, SOX2, or DCX, is carried out in a blinded fashion to minimize bias. We make cell counts across multiple brain sections, presenting the data as averages per section or hemisphere for consistency. When examining microglia, we sometimes categorize cells based on their morphology, ranging from ramified (resting state) to amoeboid (highly activated), providing insight into immune activity in the brain.

Discussion of Alternative Methods

While confocal and epifluorescence microscopy are widely used for imaging and quantification in cell biology, alternative methods such as light-sheet microscopy and two-photon microscopy offer distinct advantages. Light-sheet microscopy allows for faster imaging of large tissue volumes with minimal photodamage, making it suitable for imaging entire organoids or thick tissue sections. Two-photon microscopy, on the other hand, is ideal for deep-tissue imaging with less scattering, making it particularly useful for live tissue imaging in real time. However, these techniques often require specialized equipment and are more technically demanding. For researchers focused on cell quantification or localization, the choice between these methods depends on the balance between imaging depth, resolution, and sample preparation time.

Animal Perfusion, Tissue Processing, and Immunohistochemistry

In standard tissue preparation for histological analysis, we first deeply anesthetize the animals and perform transcardial perfusion with saline to clear blood from the vasculature, followed by perfusion with cold 4% paraformaldehyde (PFA) to fix the tissues. After perfusion, we remove the brains, post-fix them in 4% PFA overnight, and cryoprotect them in a sucrose solution to prevent freezing damage. Once cryoprotected, we freeze the brains, typically using dry ice, and section them coronally at 30 µm thickness using a microtome. We store the tissue sections at -20°C in an antifreeze solution for later use in staining procedures.

For immunohistochemistry, we wash the free-floating tissue sections and incubate them with a blocking solution to prevent nonspecific antibody binding. The sections are then incubated overnight with primary antibodies at 4°C, followed by washing and incubation with secondary antibodies. We often use a nuclear stain, such as DAPI, to visualize cell nuclei. After staining, we mount the sections on positively charged slides and cover them with a mounting medium that preserves fluorescence, such as DABCO.

Discussion of Alternative Methods

While perfusion with PFA and cryoprotection is a widely used method for preserving tissue integrity, alternative fixation methods, such as paraformaldehyde combined with glutaraldehyde, can provide better preservation of cellular structures, especially for electron microscopy. Additionally, instead of free-floating sections, some protocols use paraffin embedding, which allows for more precise cutting and long-term storage, but requires more labor-intensive deparaffinization steps before staining. When it comes to signal amplification, enzymatic amplification systems or tyramide signal amplification (TSA) can provide higher sensitivity compared to standard fluorescent antibody labeling. The choice of method depends on the desired resolution, tissue preservation needs, and the sensitivity of the detection system.

RNA Isolation for RNA Sequencing and qRT-PCR

For bulk RNA sequencing, we commonly isolate total RNA from specific cell populations, such as GFP+ sorted cells, using kits like the miRNeasy Micro Kit (Qiagen). In general, we thaw the samples, homogenize them, and subject them to phase separation using chloroform to extract RNA. We often perform on-column DNase digestion to remove any contaminating DNA, ensuring the purity of the RNA sample. The final RNA is eluted in water, and concentrations are measured using instruments like the Agilent Bioanalyzer, which provides precise quantification, particularly when working with low RNA input.

For quantitative RT-PCR (qRT-PCR), we similarly isolate total RNA using kits such as the RNeasy Mini Kit (Qiagen). This process typically involves homogenizing tissue samples or sorted cells in lysis buffer, followed by RNA purification with on-column DNase digestion. We elute the final RNA in water, and concentrations are measured using a spectrophotometer, such as the Nanodrop, which provides quick and reliable measurements of RNA concentration and purity.

Discussion of Alternative Methods

Alternative methods for RNA isolation and quantification exist, each with its own strengths. For example, TRIzol-based RNA extraction is a commonly used alternative to kit-based extractions and can be more cost-effective for large sample sets. However, TRIzol requires manual phase separation, which can be more labor-intensive and variable compared to column-based kits. For RNA quantification, Qubit fluorometry can be used as an alternative to the Nanodrop for more accurate RNA measurements, particularly in low-concentration samples, as it is less affected by contaminants such as proteins or salts. The choice of method depends on the sample type, RNA yield required, and the downstream applications.

RT-qPCR

We synthesize cDNA from RNA and use it for RT-qPCR to analyze gene expression levels. We perform RT-qPCR with a master mix and gene-specific probes, often using 96-well plates, where each well contains a reaction mixture that includes the cDNA. The process involves an initial denaturation step, followed by multiple cycles of denaturation and annealing/extension. Data is collected in real-time, and we normalize

gene expression levels to a reference gene (such as GAPDH) and compare them to control conditions. This method is widely used for accurate gene expression quantification, offering higher sensitivity and specificity compared to traditional PCR methods and can be applied across various experimental contexts.

Discussion of Alternative Methods

For gene expression analysis, RT-qPCR is a widely used method, with options such as TaqMan probes and SYBR Green. TaqMan probes are highly specific, using fluorescently labeled probes that bind to target sequences, making them ideal for studies involving complex gene families or low-abundance transcripts. In contrast, SYBR Green is a more cost-effective alternative that binds to double-stranded DNA and fluoresces during amplification. While SYBR Green offers affordability, it can be less specific, as it detects any double-stranded DNA, including nonspecific products.

When choosing between these methods, the level of specificity and budget are important considerations. TaqMan is typically preferred for more precise applications, while SYBR Green may be adequate for routine, less complex analyses. In addition, accurate normalization in qPCR is crucial, often relying on reference genes like GAPDH or ACTB, with tools such as GeNorm available to validate reference gene stability.

Alternative approaches to RT-qPCR include RNA sequencing, which offers a broader, high-throughput view of gene expression by sequencing entire transcriptomes. RNA-seq provides a more comprehensive and unbiased analysis of gene expression levels, detecting both known and novel transcripts, but it is more resource-intensive and may not be necessary for targeted gene studies. Another method is digital PCR (dPCR), which offers absolute quantification without the need for reference genes, providing high sensitivity and precision, particularly in detecting low-abundance targets. Each method has its advantages, and the choice depends on the required resolution, cost, and the scope of the study.

Library preparation and RNA sequencing

For single-cell RNA sequencing, we generate libraries from individual cells using the 10x Genomics' Chromium system. Cells of interest can be sorted by specific markers and processed according to standard protocols. The workflow typically includes cDNA amplification and sample indexing before sequencing on platforms such as the Illumina

series. The number of cells sequenced per sample varies, often ranging from a few hundred to several thousand, depending on the study design, with samples sometimes pooled from multiple individuals to account for biological variability.

For bulk RNA-seq, we prepare libraries from total RNA, providing an averaged gene expression profile across all cells in the sample. We use kits like the SMARTer Stranded Total RNA-Seq Kit, which can handle low RNA inputs. The RNA is fragmented and amplified through PCR, and the libraries are sequenced on high-throughput platforms such as the Illumina NextSeq.

Discussion of Alternative Methods

Bulk RNA-seq provides an averaged gene expression profile across all cells in a sample, while single-cell RNA-seq allows for the exploration of heterogeneity at the individual cell level, making it ideal for detailed cellular studies. Single-cell RNA sequencing typically has lower sequencing depth per cell compared to bulk RNA-seq, making it easier to miss differentially expressed genes, particularly low-abundance ones. Each method has its advantages, with the choice depending on the study's focus on individual cell variation versus overall gene expression patterns.

There are several alternatives to the Chromium system from 10x Genomics for single-cell RNA sequencing, each offering distinct advantages depending on research needs. Chromium is ideal for high-throughput studies, capable of sequencing thousands of cells in a single experiment, making it perfect for large-scale projects focusing on cell population diversity. However, methods like Smart-seq provide more detailed, full-length transcript coverage, which is particularly useful for capturing transcript isoforms. Smart-seq offers higher sensitivity and resolution but is better suited for smaller-scale studies due to its higher per-cell cost and labor-intensive process.

Drop-seq is a cost-effective, high-throughput alternative, similar to Chromium in scale but slightly less sensitive due to its simpler droplet-based technology. While Drop-seq offers a more affordable option for large studies, it may lack the precision and depth of transcript coverage that Smart-seq can provide. Smart-seq, though more precise in capturing the full transcriptome, has lower throughput, making it suitable for experiments requiring detailed transcript analysis on a smaller scale.

For bulk RNA sequencing, TruSeq RNA Library Prep from Illumina is widely used for its straightforward and efficient workflow, particularly for standard RNA inputs. When dealing with low-input RNA, the SMARTer Stranded Total RNA-Seq Kit is often preferred, as it ensures better performance with minimal RNA.

Long-read sequencing platforms, such as Oxford Nanopore and PacBio, provide full-length transcript reads, which are particularly valuable for studying complex transcript isoforms or RNA modifications. These technologies offer deeper insights into the transcriptome by capturing long reads without the need for assembly, but they come with higher costs, lower throughput, and require more advanced bioinformatics analysis.

Spatial transcriptomics techniques like 10x Genomics' Visium and Slide-seq add a new layer of information by integrating spatial context into gene expression data. Visium uses spatially barcoded oligonucleotide probes embedded in a slide to capture mRNA from tissue sections, offering spatial resolution of around 50-100 microns, making it ideal for studying broad tissue architecture. However, its spatial resolution is not fine enough to capture single-cell resolution. Slide-seq, which uses beads with spatial barcodes to capture mRNA from the tissue surface, offers higher spatial resolution (approximately 10 microns), allowing for near single-cell resolution, but with a trade-off in sensitivity compared to techniques like Smart-seq.

These spatial techniques provide a more comprehensive view of tissue organization and cellular interactions, but they require more specialized equipment and expertise. MERFISH (Multiplexed Error-Robust Fluorescence In Situ Hybridization) is another spatial technique, providing single-molecule, single-cell spatial resolution but with more labor-intensive protocols and requiring predefined gene panels.

In summary, the choice of technique depends on the specific needs of the study. Chromium and Drop-seq are excellent for high-throughput studies, while Smart-seq is better for detailed, full-length transcript coverage in smaller experiments. Long-read platforms like Nanopore and PacBio are ideal for isoform-level resolution but at a higher cost. For spatial information, Visium provides broad tissue-level insights with moderate resolution, while Slide-seq and MERFISH offer higher spatial resolution, with Slide-seq balancing throughput and cost, and MERFISH offering the most detailed spatial data but with more labor-intensive preparation. Each method presents a trade-off between resolution, cost, throughput, and the complexity of analysis.

Bioinformatics analyses

Raw Data Processing and Quality Control

We typically process single-cell RNA sequencing data through standardized pipelines, such as Cell Ranger, to generate count matrices. These matrices are then

loaded into computational environments, like R, for further analysis. We use tools like DropletUtils and Scater to normalize cell counts, while Seurat is a widely used package for managing and analyzing single-cell data. Our quality control steps involve removing low-quality cells based on the number of detected genes and filtering out cells with high levels of mitochondrial or ribosomal genes, which often indicate poor-quality data.

For bulk RNA sequencing, we use tools like HISAT2 for aligning reads to reference genomes, and we assess quality control metrics with software such as FastQC and Picard. Following alignment, we perform transcript assembly and gene expression quantification using tools like StringTie.

Clustering and Cell Type Assignment

Once we have processed and cleaned the data, we apply clustering methods to group similar cells based on gene expression. This typically involves identifying highly variable genes and performing principal component analysis (PCA) to reduce dimensionality. We use common algorithms like Louvain clustering to assign cells to clusters, which are then visualized using techniques such as uniform manifold approximation and projection (UMAP). We assign cell types by comparing clusters to reference datasets and use known markers for specific cell types (e.g., Gfap for astroglia, Dcx for neuroblasts) to fine-tune our annotations. Unwanted clusters, such as those corresponding to non-target cells, may be discarded to focus on the relevant cell populations.

Differential Gene Expression Analysis and Quantification

For differential gene expression analysis, we use tools like DESeq2 for bulk RNA sequencing or FindMarkers in Seurat for single-cell data to compare gene expression between conditions. We typically filter the results for significance using adjusted p-values, employing methods such as Bonferroni correction or false discovery rate (FDR) adjustments to account for multiple testing. Data visualization is an essential part of our analyses, where we use tools like ggplot2 to generate boxplots and heatmaps to highlight gene expression patterns. To understand the biological processes associated with differentially expressed genes, we frequently apply gene ontology (GO) term enrichment, often using programs like ClusterProfiler for this task.

Discussion of Alternative Methods

There are several alternative methods available for processing and analyzing RNA sequencing data. For instance, normalization approaches like scran's quickCluster and computeSumFactors are used in this workflow, but methods like SCTransform, available in Seurat, provide alternatives that automatically handle normalization and variance stabilization. For differential gene expression, limma or edgeR can serve as alternatives to DESeq2 in bulk RNA sequencing studies, offering different statistical approaches depending on the size and nature of the dataset. Each method comes with trade-offs; for instance, limma is known for speed and handling small datasets effectively, while DESeq2 excels with larger and more complex datasets.

Ultimately, choosing between these methods often depends on the specific needs of the experiment, the dataset size, and computational resources available. As bioinformatics continues to evolve, there is increasing flexibility in tailoring pipelines to specific research needs. In addition, there are several web-based applications, like ScarfWeb, that allow users to perform analysis without programming expertise.

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Paper I

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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 CXCR5/5/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*[10] and levels of CXCL13 in CSF increase in some neurological disorders such as multiple sclerosis.[11] Interestingly, *Cxcr5* has also been suggested to regulate neurogenesis in adult zebra fish[12] and in the murine DG.[13]

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.

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.

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 ab 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 & laboratorier, 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 $KH_2PO_4{\cdot}H_2O$, 16.03 g/L $K_2HPO_4{\cdot}2H_2O$, 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 (TKPBS) (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 at RT for 2 hours, followed by nuclear staining with Hoechst (Thermo Fisher Scientific, 1:1000) in KPBS for 10 minutes. Sections were then mounted on slides, briefly rinsed in ddH_2O, dried, and finally coverslipped using DABCO. Staining of free-floating sections was performed on an orbital shaker, attached sections in a humidified chamber and all washes were performed 3×5min with KPBS.

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

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% H_2O_2 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 subdissected 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 Biotech, 130-107-677) following manufacturer's guidelines. Cells were then purified through a two-step gradient as previously described[15] 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% CO_2, and 37°C. NSPCs from SVZ were expanded, as described[15] 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 H_2O to a final volume of 60 µL. Concentrations was 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 IQ5 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)[16] 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 uniformmanifold 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.[13] 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,[17] the increased inflammation in aged brain[18] together with increased CXCL13 levels during brain inflammation[11] 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

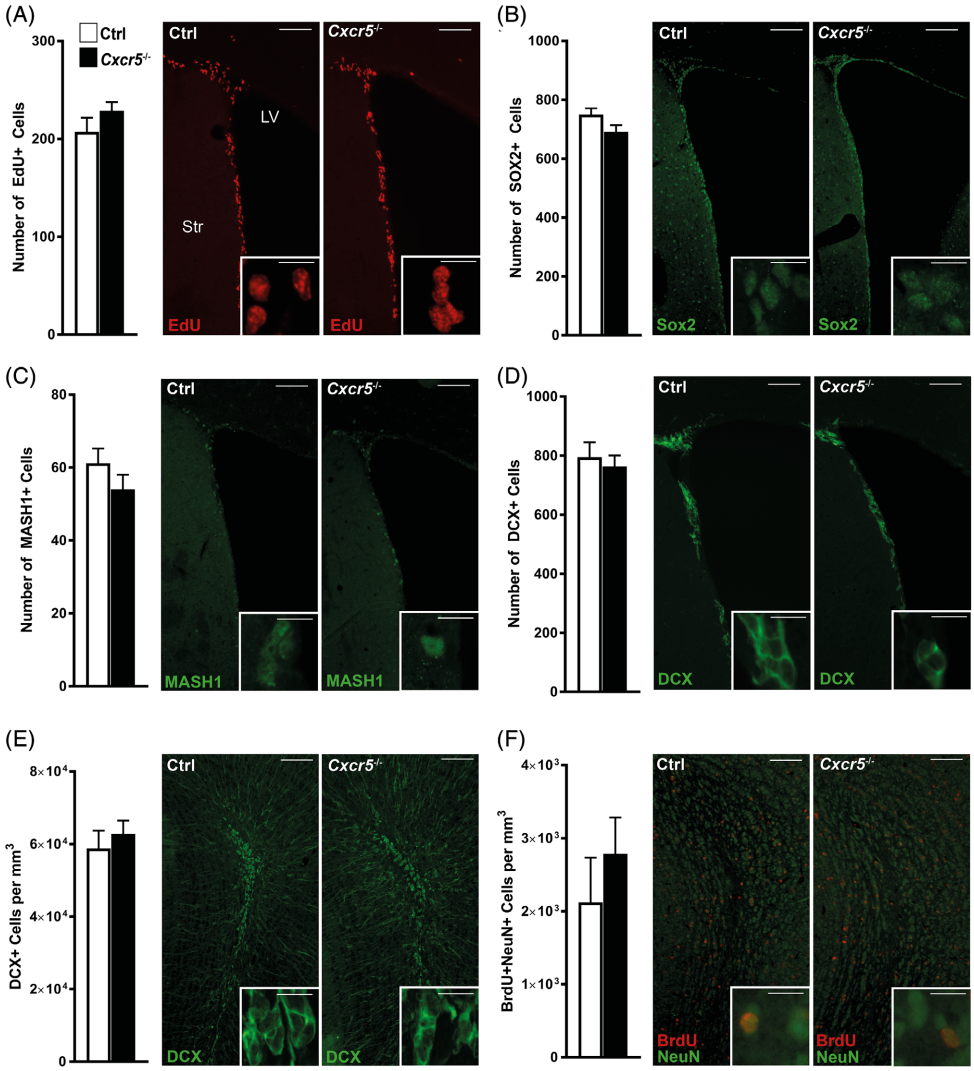

FIGURE 1 Adult SVZ neurogenesis is not affected by loss of *Cxcr5*. Quantification and representative (overview and high magnification) images of, A, BrdU+, B, DCX+, C, Mash1+, and, D, SOX2+ cells, in the SVZ of 3-month-old wild type (WT) and *Cxcr5*$^{-/-}$ mice. Quantification and representative (overview 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/mm^3) 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

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

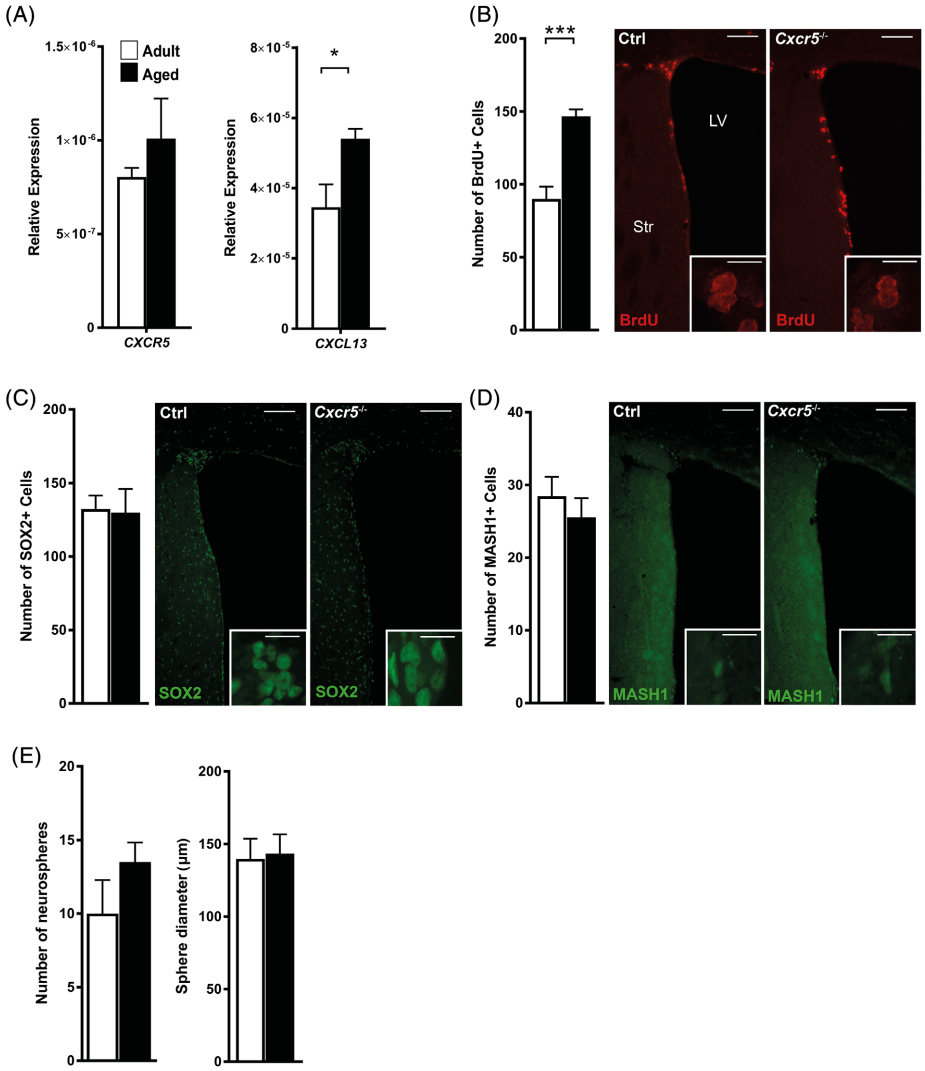

FIGURE 2 Germline loss of *Cxcr5* leads to increased SVZ proliferation in the aged brain. A, mRNA expression as assessed by real time quantitative polymerase chain reaction (RT-qPCR) of *Cxcr5* and *Cxcl13* in whole brain tissue from 4- and 24-month-old wild type (WT) mice. Quantification and representative (overview and high magnification) images of, B, BrdU+, C, SOX2+, and, D, Mash1+ cells, in the SVZ of 18-month-old WT and *Cxcr5*$^{-/-}$ mice. E, Total number and size of primary neurospheres derived from the SVZ of 20-month-old *Cxcr5*$^{-/-}$ and WT mice at 7 days in vitro. A-C, Data presented as means per 30 µm sections and, D, total number and diameter (µm) ± SEM; n = 5 (A-C) and n = 4 (D). **P* < .05, with two-tailed unpaired *t* test. Scale bar = 100 µm (overview), 10 µm (high magnification). SVZ, subventricular zone

or more proliferative NSPC population, we next examined the NSPC compartment in the SVZ of WT and *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 *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

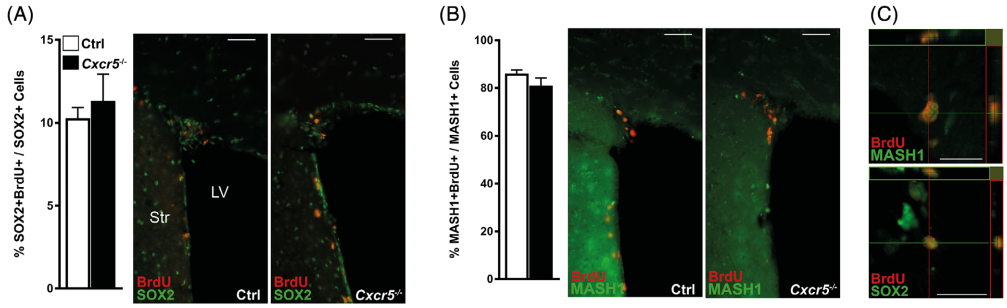

FIGURE 3 Loss of *Cxcr5* does not affect proliferation of Sox2+ and Mash1+ NSPC populations. Quantification and representative images of, A, SOX2+/BrdU+ and, B, Mash1+/BrdU+ cells out of total number of SOX2+ and Mash1+ cells, respectively. C, Orthogonal projection confirming BrdU colocalization with SOX2 and Mash1. Data presented as mean ratio (%) per 30 μm sections ± SEM; n = 5. $^{*}P < .05$, $^{***}P < 0.001$, with two-tailed unpaired *t* test. Scale bar = 50 μm (A, B), 20 μm (C). NSPCs, neural stem and progenitor cells

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 the SVZ. Surprisingly, we detected significantly lower number of SVZ neuroblasts in the aged *Cxcr5*$^{-/-}$ as compared to control animals (Figure 4B).

During normal neurogenesis, neuroblasts migrate out of the SVZ through the RMS and eventually arrive to the OB. Given the role of CXCR5 signaling in migration of immune cells,[11] we next wanted to assess whether the decrease in SVZ neuroblasts could be due to increased migration out of the SVZ. We 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

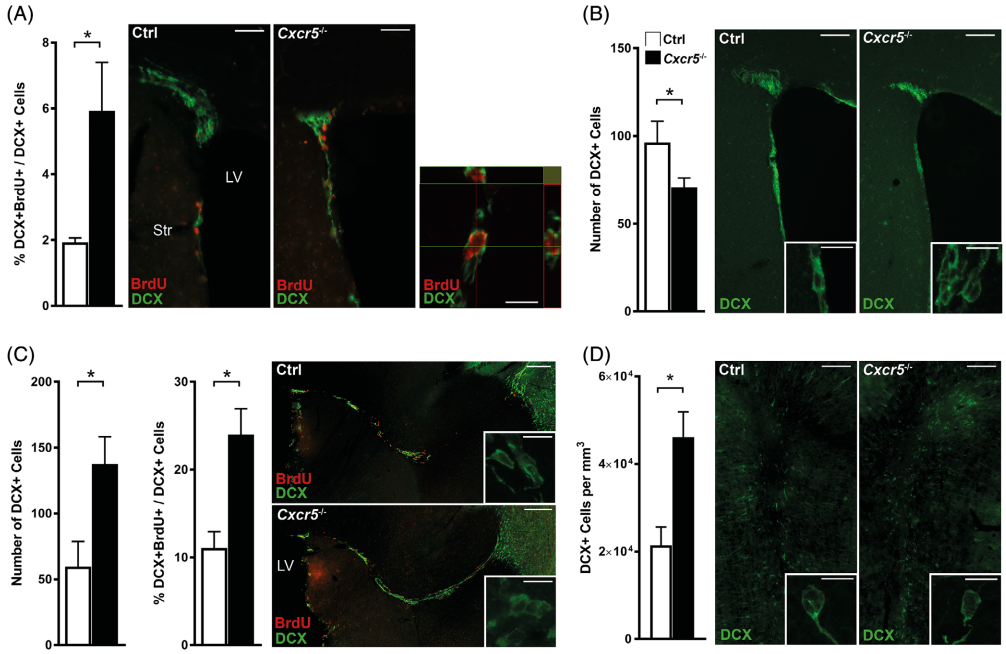

FIGURE 4 Loss of *Cxcr5* increase neuroblast proliferation and migration. A, Quantification (left) and representative images (middle) of DCX+/BrdU+ cells in SVZ of 18-month-old WT and *Cxcr5*$^{-/-}$ mice, and orthogonal projection confirming BrdU colocalization with DCX (right). B, Quantification and 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/mm^3) 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

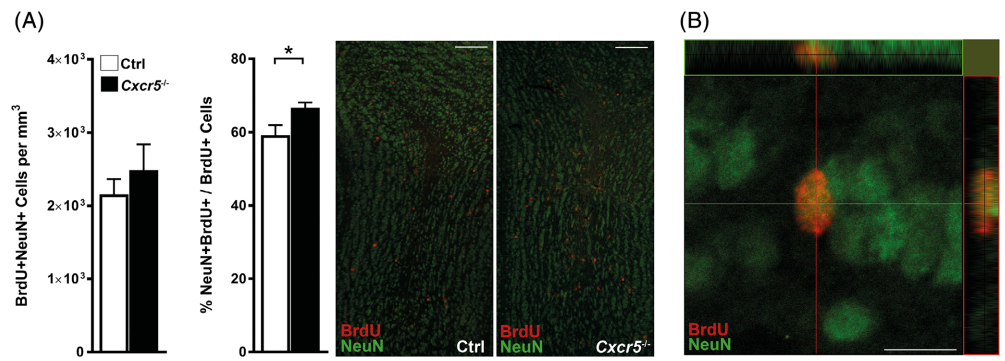

FIGURE 5 Number of newly formed neurons in the OB is not affected by loss of *Cxcr5*. A, Total number (left), percentage (middle), and representative images (right) of NeuN+/BrdU+ cells in the OB (GCL and IPL) of 18-month-old WT and *Cxcr5*$^{-/-}$ mice. B, Orthogonal projection confirming BrdU colocalization with NeuN. A, Data presented as mean cell density (cells/mm^3) per 20 μm section and, B, mean percentage per 20 μm section ± SEM; *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

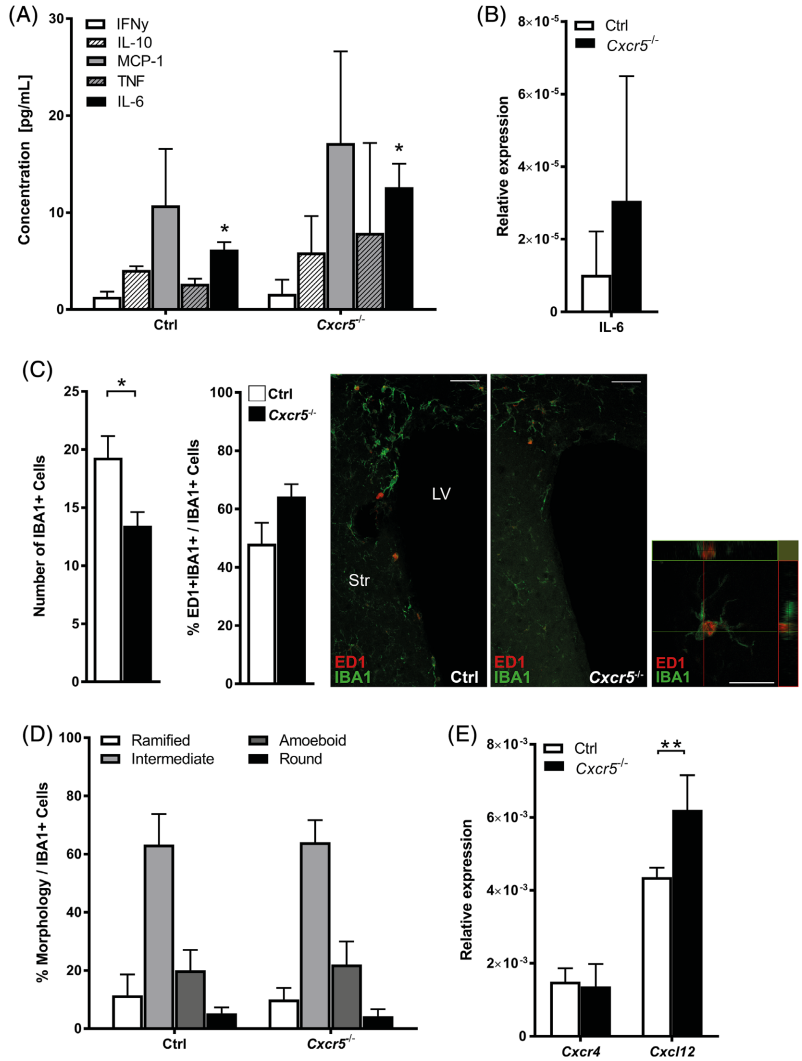

FIGURE 6 Loss of *Cxcr5* leads to increased levels of circulating IL-6, and Cxcl12 expression in SVZ. A, Cytometric bead array analysis of circulating cytokine concentrations in plasma from 20-month-old wild type (WT) and $Cxcr5^{-/-}$ mice. B, mRNA expression as assessed by real time quantitative polymerase 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 Iba + 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.[16] 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 CXCR5 in immune cells and that inflammation is a potent regulator of neurogenesis,[17] 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 *Cxcr5*$^{-/-}$ mice using a cytometric bead array, without detecting any differences.[13] 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*$^{-/-}$ as compared to control mice, but otherwise no differences could be observed (Figure 6A). Because IL-6 has been shown to increase proliferation of NSPCs,[22] 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.[23] We therefore 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-, through intermediate-, to amoeboid- and finally round morphology.[24] 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 S4A,B and 6C,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.[25] Neuroblasts use CXCR4 and its ligand CXCL12 for migration in the brain and CXCL12 has been suggested to promote proliferation of *Cxcr4* expressing cells.[26-28] 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.[16] This analysis revealed, in agreement with previous studies,[29] 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 reduced neuroblast proliferation[33] 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 mileu, 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 zebra fish brain.[12] In contrast to findings from zebra fish, 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 mice[37,38] 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 undistinguishable 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.[42] 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.[45] 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

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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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of this article.

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RESEARCH ARTICLE

WILEY

Microglia undergo disease-associated transcriptional activation and CX3C motif chemokine receptor 1 expression regulates neurogenesis in the aged brain

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Abstract

Adult neurogenesis continues throughout life but declines dramatically with age and in neurodegenerative disorders such as Alzheimer's disease. In parallel, microglia become activated resulting in chronic inflammation in the aged brain. A unique type of microglia, suggested to support neurogenesis, exists in the subventricular zone (SVZ), but little is known how they are affected by aging. We analyzed the transcriptome of aging microglia and identified a unique neuroprotective activation profile in aged SVZ microglia, which is partly shared with disease-associated microglia (DAM). CX3C motif chemokine receptor 1 (CX3CR1) is characteristically expressed by brain microglia where it directs migration to targets for phagocytosis. We show that Cx3cr1 expression, as in DAM, is downregulated in old SVZ microglia and that heterozygous Cx3cr1 mice have increased proliferation and neuroblast number in the aged SVZ but not in the dentate gyrus, identifying CX3CR1 signaling as a novel age and brain region-specific regulator of neurogenesis.

KEYWORDS
aging, CX3C motif chemokine receptor 1, disease-associated microglia, microglia, neurogenesis, subventricular zone

1 | INTRODUCTION

Neurogenesis, the formation of new neurons from neural stem cells (NSCs), occurs in two adult neurogenic niches of the mammalian brain, the subgrandular zone (SGZ) of the dentate gyrus (DG) in the hippocampus, and the subventricular zone (SVZ) lining the lateral ventricles. Adult NSCs in both neurogenic niches are largely maintained in a quiescent state, upon activation, they self-renew and/or differentiate to produce proliferative intermediate progenitors, which in turn generates migrating neuroblasts. SVZ neuroblasts migrate via the rostral migratory stream into the olfactory bulb (OB) where they develop into different types of interneurons, whereas SGZ neuroblasts migrate only a short distance to the granule cell layer within the DG before differentiating into granule neurons (Toni et al., 2008; Zhao et al., 2006).

Although neurogenesis continues throughout life, the level declines drastically with age. NSC number drops and NSC as well as progenitor proliferation and the formation of new neurons decrease during aging (Ahlenius et al., 2009). In addition, neurogenesis declines even further in age-related neurodegenerative disorders such as Alzheimer's disease (Moreno-Jimenez et al., 2019).

Microglia, the brain-resident macrophages, are heavily involved in regulating adult neurogenesis (Kreisel et al., 2019; Ribeiro Xavier et al., 2015). Commonly found in a resting state, microglia can undergo different activations depending on environmental cues. In vitro studies indicate that anti-inflammatory activation supports, whereas a pro-inflammatory state opposes neurogenesis (Kettenmann et al., 2011; Walton et al., 2006). In addition, microglia can enter alternative activation states. For instance, in mouse models of Alzheimer's disease, microglia acquire a neuroprotective phenotype, known as disease-associated microglia (DAM), that prevents neurodegeneration by active clearance of protein aggregates and amyloid proteins (Deczkowska et al., 2018; Keren-Shaul et al., 2017).

Within the neurogenic niches, microglia have specialized phenotypes. In the DG, microglia display a hyper-ramified morphology. They play a dual role by promoting neuronal differentiation and neuroblast survival, and by phagocytizing apoptotic newborn neurons (Diaz-Aparicio et al., 2020; Kreisel et al., 2019; Sierra et al., 2010). On the other hand, SVZ microglia have enlarged cell soma and fewer processes, usually characteristic of activated and phagocytizing cells. Despite their morphology, SVZ microglia display a resting phenotype, are rarely involved in phagocytosis, and have therefore been suggested to support neurogenesis (Ribeiro Xavier et al., 2015).

Microglial cells, globally and in neurogenic niches, highly and exclusively express the CX3C motif chemokine receptor 1 (CX3CR1) (Ransohoff, 2007; Xavier et al., 2015). In the hippocampus, CX3CR1 promotes the differentiation of NSCs to neuroblasts (Bachstetter et al., 2011; Rogers et al., 2011). However, the role of CX3CR1 in SVZ neurogenesis and during aging is unclear. In addition, in the SVZ, microglia are heterogenous, and many lack expression of the common microglia marker ionized calcium-binding adaptor molecule 1 (IBA1(Aif1)) (Ribeiro Xavier et al., 2015), which plays key roles in activated microglia (Ohsawa et al., 2000, 2004).

During aging, IBA1+ microglia undergo progressive chronic activation in the SVZ and have been suggested to contribute to the reduction in neurogenesis (Solano Fonseca et al., 2016). However, age-related changes in microglia with the potential to affect SVZ neurogenesis have not been studied in great detail.

Here we analyzed the transcriptome of aging microglia and identified a unique neuroprotective activation profile in aged SVZ microglia, which is partly shared with DAM. We show that Iba1(Aif1) positive and negative microglia acquire similar aging signatures and that Cx3cr1 expression is down-regulated in old SVZ microglia. Furthermore, we show that heterozygous Cx3cr1 mice have increased proliferation and neuroblast number in the aged SVZ, identifying CX3CR1 signaling as a novel regulator of neurogenesis in the aging brain.

2 | MATERIALS AND METHODS

2.1 | Single-Cell RNA sequencing data analysis

For transcriptome analysis of SVZ microglia, we used a previously published dataset containing the normalized and quality-controlled 10× single-cell RNA sequencing data of young (3 months) and old (28–29 months) mouse SVZ (BioProject: PRJNA450425) (Dulken et al., 2019). For analysis of DAM, previously published data with differentially expressed genes (DEGs) between homeostatic and DAM was used (Keren-Shaul et al., 2017) (raw data, GEO: GSE98969), and for analysis with whole brain microglia, we used previously published data with DEGs between young (2–3 months) and old (21–22 months) mouse brain microglia (Ximerakis et al., 2019) (raw data, GEO: GSE129788). Data were analyzed using Seurat (4.1.0) in R computational environment (4.1.2). Highly variable genes (HVGs) were identified with the variance stabilizing transformation method. Principal component analysis was run on the HVGs and the first 22 principle components used for clustering and visualization with uniform manifold approximation and projection. Default clustering algorithm was used in Seurat with k parameter set to 6 and resolution to 0.5. Clusters were classified using singleR (1.8.1) based on a reference mouse brain dataset (Benayoun et al., 2019), and clusters annotated as microglia were used for further analysis. DimPlot (Seurat) was used to illustrate clusters, FeaturePlot (Seurat) to illustrate single-cell gene expression, RidgePlot (Seurat) for ridge plot, VlnPlot (Seurat) for violin plots, and for comparison of DEG between datasets, we employed ggvenn (0.1.9).

To study Aif1(Iba1) positive and negative microglia, we divided SVZ microglia into groups based on log-normalized expression of Aif1 above or below 0.5 before conducting differential gene expression analysis. Differential gene expression analysis was carried out with findMarkers (Seurat) function, considering p-values based on Bonferroni correction, using all genes in the dataset, (p_val_adj) <.05 as significant, and DoHeatmap (Seurat) for heatmaps. Gene ontology (GO) terms were identified for DEGs with adjusted p-values <.05 using the enrichGO function of ClusterProfiler (4.2.2) with minGSSize 1, maxGSSize 500 and q- and p-value cutoff set to 1.

2.2 | Mice

All animal experiments were approved by Malmö-Lund Ethical Committee and performed in accordance with the EU directive regarding animal rights. All histological studies were performed on a mix of male and female Cx3cr1$^{+/+}$, Cx3cr1$^{+/Egfp}$ mice on C57BL/6J background (Jackson;

005582) (Jung et al., 2000) in the age groups 3–4 months and 17–19 months. All animals used were bred and housed in the animal facility at the Lund University Biomedical Center with ad libitum access to food and water. The vivarium was maintained under controlled conditions at 22˚C, 40%–60% humidity, and a 12 h diurnal cycle.

2.3 | Genotyping

DNA was extracted from biopsy samples, collected from pups during ear marking, using the KAPA Mouse genotyping kit (KAPA Biosystems; KK7103) and its recommended protocol (per 100 μL reaction—88 μL RNase-free H20, 10 μL 10× Kapa Express Extract Buffer, 2 μL KAPA Express Extract Enzyme, 2 mm Mouse Tissue; 75˚C 10 min, 95˚C 5 min). Specific sequences of the Cx3cr1 gene and the Egfp insert were amplified using a Hot Start Touchdown PCR protocol (Primers in Table S1; Recipe in Table S2; 94˚C 2 min, 10 cycles [94˚C 20 s, 65˚C −0.5˚C/cycle 15 s, 68˚C 10 s], 28 cycles [94˚C 15 s, 60˚C 15 s, 72˚C 10 s], 72˚C 2 min). PCR products were resolved by electrophoresis on a 2% agarose gel at 90 V for 50 min with a 100 bp ladder (Thermo Fisher Scientific; 15628050).

2.4 | Immunohistochemistry

Animals were deeply anaesthetized with pentobarbital (Apotek Produktion & Laboratorier, APL; 338327) injection and transcardially perfused with 0.9% w/v saline followed by 4% cold paraformaldehyde (PFA). The brains were removed, postfixed overnight in 4% PFA at 4˚C, and cryoprotected in 20% sucrose, in phosphate buffer (PB; 0.2 M $NaH_2PO_4{\cdot}H_2O$, 0.2 M $Na_2HPO_4{\cdot}2H_2O$). The cerebellum and OB were discarded, and cerebrums were frozen on dry ice, cut coronally at 30 μm thickness using a microtome (Leica Biosystems), and stored at −20˚C in an antifreeze solution (30% v/v ethylene glycol, 30% v/v glycerol, PB).

During staining, free-floating sections were washed thrice, 5 min each time, with potassium PB saline (KPBS; 2.745 g/L $KH_2PO_4{\cdot}H_2O$, 16.03 g/L $K_2HPO_4{\cdot}2H_2O$, 50.4 g/L NaCl), followed by a 60 min incubation at room temperature with a blocking solution containing 5% normal donkey serum in 0.25% Triton KPBS (TKPBS; 0.25% v/v Triton X-100, KPBS). Next, sections were incubated with primary antibodies (Table S3) diluted in blocking solution, overnight at 4˚C. After primary antibody incubation, sections were washed, 5 min each time, twice with 0.25% TKPBS, and once with blocking solution before incubation with secondary antibodies (Table S3), diluted in blocking solution, for 120 min at room temperature and protected from light. The incubation was followed by a nuclear staining with 4′,6-diamidino-2-phenylindole solution (DAPI; diluted 1000× in KPBS) for 10 min at room temperature and protected from light. Sections were washed thrice, 5 min each time, with KPBS at room temperature before mounting them on slides with a positively charged coating (5 g/L gelatin, 0.5 g/L $KCrS_2O_8$, Milli-Q H_2O). Mounted slides were covered with 2.5% 1,4-diazabicyclo[2.2.2]octane (DABCO) mounting media (25 g/L DABCO, 24% w/v glycerol, 9.6% w/v polyvinyl alcohol, 96 mM Tris–HCl at pH 8–8.5, dH_2O) and a coverslip.

Alternatively, after secondary antibody incubation, staining with minichromosome maintenance 2 (MCM2) underwent signal amplification with biotinylated antibodies (Table S3) for 30 min at room temperature. Sections were then washed thrice, 5 min each time, with KPBS, followed by 5 min incubation in blocking solution and 90 min incubation with conjugated streptavidin (Table S3) in blocking solution at room temperature. Finally, prior to DAPI staining, the sections were washed for 5 min with KPBS at room temperature. All steps are protected from light.

2.5 | Antibody characterization

The doublecortin (DCX) antibody (Aves Labs, AB_2313540, used at 1:2000) was raised in chicken against synthetic peptides corresponding to different regions of the DCX gene product but shared between the human (CAA06617.1, NCBI) and mouse (AAT58219.1, NCBI) sequences. It has a molecular weight of 40 kD (manufacturer's datasheet) and stains a pattern of cellular morphology and distribution in the mouse brain that is identical with previous reports (Gupta et al., 2021). We validated staining in Dcx-green fluorescent protein (GFP) mice, which revealed a high overlap with Dcx and GFP.

The GFP antibody (Merck Millipore, AB16901, used at 1:500) is raised in chicken and made against native GFP from the jellyfish Aequorea victoria. It detects a band of 30 kD (manufacturer's datasheet) and stains mouse brain cells transfected with a plasmid directing expression of GFP (Van Kampen et al., 2014). We validated staining in Dcx-GFP mouse brain section where cells without GFP expression showed no staining.

The ionized calcium-binding adapter molecule 1 (IBA1) antibody (Fujifilm WAKO Chemicals, 019-19741, used at 1:500), raised in rabbit against a synthetic peptide corresponding to the Iba1 carboxy-terminal sequence, which was conserved among human, rat, and mouse Iba1 protein sequences. It has a molecular weight of 17 kD (manufacturer's datasheet) and stains a pattern of cellular morphology and distribution in the mouse brain that is identical with previous reports (Mori et al., 2000). We validated the antibody in Iba1-GFP mice that showed a high overlap of Iba1 staining and GFP signal.

The MCM2 antibody (Cell signaling technologies, 3619s, used at 1:500) raised in rabbit was generated using MCM2 as

the antigen. It has a molecular weight of 125 kD (manufacturer's datasheet) and stains a pattern of cellular morphology and distribution in the mouse brain that is identical with previous reports (Favaloro et al., 2022). We validated the antibody by examining staining pattern in highly proliferative brain regions such as SVZ and DG.

2.6 | Imaging and quantification

All quantifications were performed blinded under an Olympus BX61 epifluorescence microscope using Olympus VS-ASW 2.9 software. Coordinates for sections on the coronal plane, for analysis, were inferred by comparing the cytoarchitecture under DAPI staining to adult mouse brain atlas (Franklin & Paxinos, 2013). All analyses were performed on sections between 0.14 and 1.1 mm from bregma for SVZ and between −1.5 and −2.25 mm from bregma for DG, in 2–6 sections per animal.

The total numbers of positive cells for each marker were counted on either hemisphere of each section in the SVZ and DG, respectively. In the SVZ, defined as the cell-dense area (in DAPI staining) on the wall of lateral ventricles, the areas were calculated. Images of co-localization of markers in cells and other high magnification images were acquired with a 63× objective in a Zeiss LSM 780 confocal microscope using Zen software.

2.7 | Statistic analysis

Cell counts for each brain hemisphere of each section in the SVZ were normalized to the respective areas to get cell density. Among hemispheres of each section, the mean cell density for SVZ and mean cell nr for DG were calculated for each animal and used as input in the statistical analysis. To test cell type occurrence between age groups in the sequencing data, ratios were calculated for each animal ($n = 3$) before running the statistical test. For comparisons between two groups, an unpaired two-tailed *t*-test was performed using GraphPad Prism 9 (GraphPad Prism Software). Results were considered significant when p-value <.05. All data are presented as mean (cells per mm^2) ± SEM.

3 | RESULTS

3.1 | *Iba1* positive and negative microglia have similar transcriptional profiles in the aged SVZ

In the SVZ, microglia have certain characteristics suggested to support neurogenesis. For instance, a significant fraction of Cx3cr1 expressing SVZ microglia lacks the common microglia marker IBA1(Aif1) (Ribeiro Xavier et al., 2015). However, whether the Iba1 negative subpopulation of microglia changes with age and if this is linked to the decreased neurogenesis observed during aging is not known. In order to investigate transcriptional changes in Iba1 positive and negative microglia in the SVZ during aging, we took advantage of a previously published and processed single-cell RNAseq dataset (Dulken et al., 2019).

We started by clustering all cells, unsupervised, with high resolution (Figure 1a), and separated microglia based on gene expression patterns defined in previous sequencing studies (Figure 1b) (Benayoun et al., 2019). As expected microglial clusters expressed the common microglia markers Cx3cr1, Iba1(Aif1), Tmem119, P2ry12, Hexb, and Fcrls (Figure 1c–h) and lacked expression of Mrc1, Cd44, and Cd3d, markers for monocytes, macrophages and T-cells, respectively (Figure 1i–k) (Jurga et al., 2020). Next, we separated microglial cells based on Iba1(Aif1) expression to acquire microglia that were either Iba1+ or Iba1− (Figure 2a) and performed differential gene expression analysis between Iba1+ and Iba1− cells for each age group individually.

Surprisingly, old Iba1− revealed very little changes compared to old Iba1+ cells (Figure 2b). Further, we investigated potential age differences in the Iba1+ and Iba1− populations separately. As expected, we found a complete overlap of DEGs in Iba1− microglia as compared to DEGs in Iba1+ microglia (Figure 2c). In addition, we highlighted Iba1 positive and negative microglia in previously acquired clusters, which illustrated their even distribution among the cell state changes that occur in aged SVZ microglia (Figure 2d).

We then calculated the ratio of Iba1− microglia for each animal and performed a *t*-test between age groups. We were not able to detect any changes in population size with age based on mRNA expression from single-cell sequencing (Figure 2e). To investigate IBA1 positive and negative microglia based on protein expression in vivo, we used IBA1 immunohistochemistry in brain sections from young and old Cx3cr1-GFP microglia reporter mice (Jung et al., 2000). As expected, we could detect GFP+ microglia in the aged SVZ, which were either Iba1 positive or negative. Quantification confirmed our findings from bioinformatics analysis that the Iba1− population does not change in size during aging (Figure 2f).

Taken together, we found no evidence for any substantial transcriptional change in Iba1− compared to Iba1+ SVZ microglia, and the population size does not change with age. Therefore, it is unlikely that Cx3cr1+/ Iba1− microglia would have any substantial impact on the neurogenic decline with age.

3.2 | Aged SVZ microglia acquire unique DAM-like neuroprotective activation

Having established that Iba1 negative microglia most likely do not play a part in the reduction in neurogenesis during aging

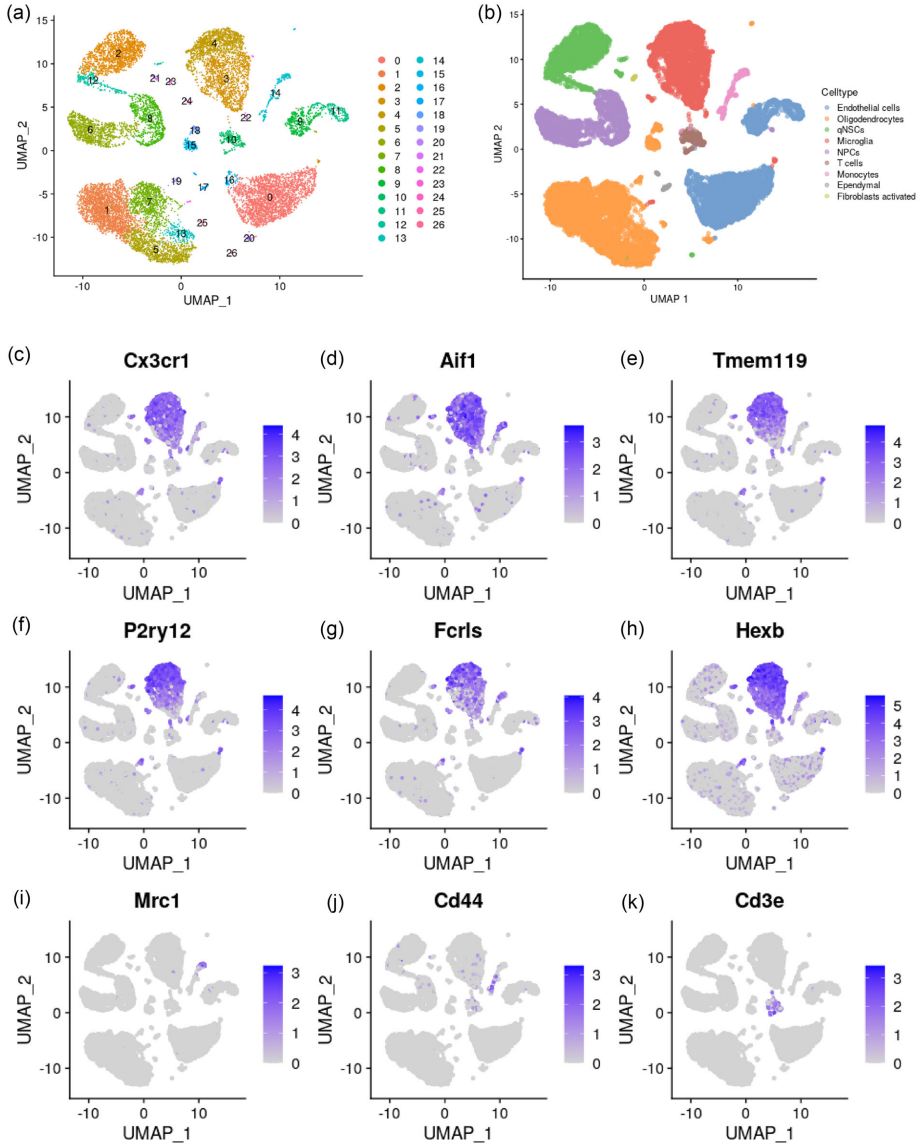

FIGURE 1 **Identification of microglial populations in the subventricular zone (SVZ)**. Uniform manifold approximation and projection (UMAP) plot showing cluster number (a), cell type annotation (b), common microglia markers CX3C motif chemokine receptor 1 (Cx3cr1) (c), Aif1 (d), Tmem119 (e), P2ry12 (f), Fcrls (g) and Hexb (h), a marker for monocytes, Mrc1 (i), a marker for macrophages, Cd44 (j), and a marker for T-cells, Cd3e (k) in the SVZ.

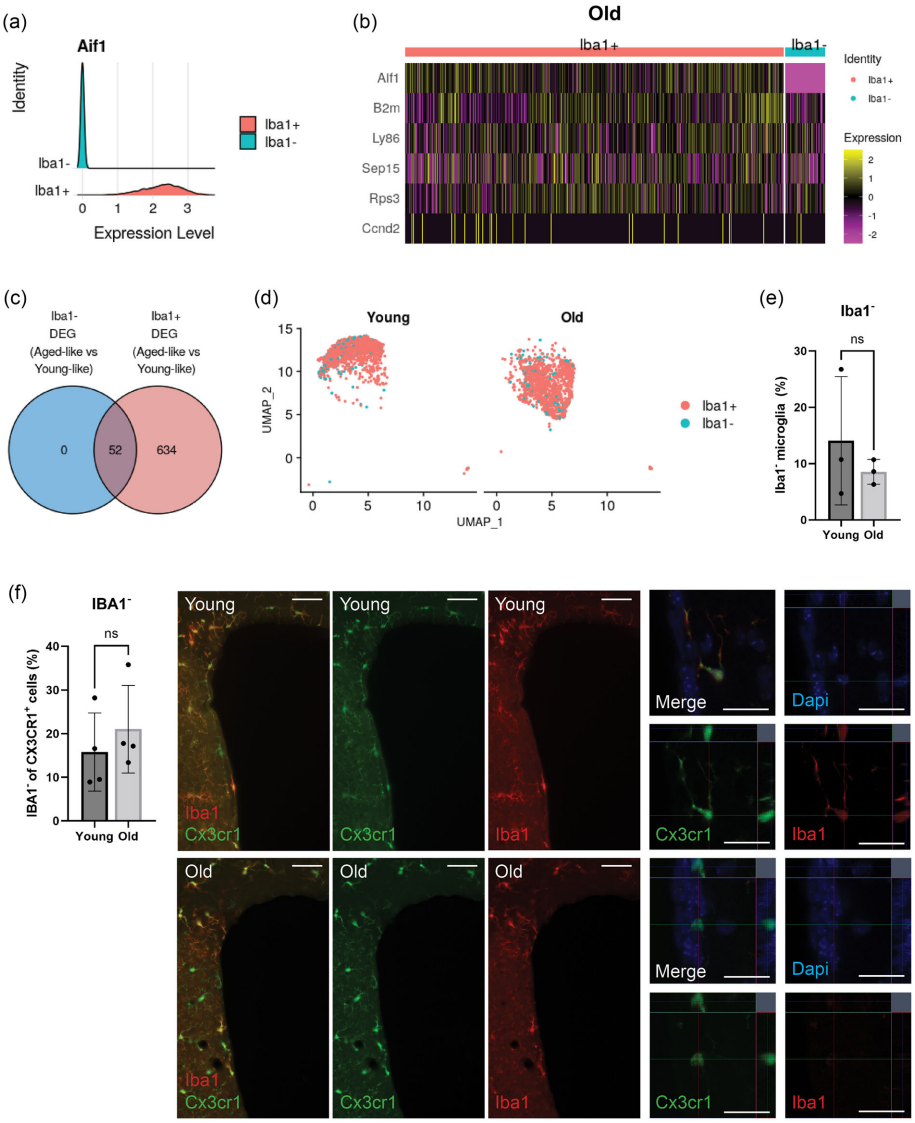

FIGURE 2 **Iba1 positive and negative microglia have similar transcription profiles in the aged subventricular zone (SVZ).** Ridgeplot showing expression levels of Aif1(Iba1) in Iba1+ and Iba1− microglia (a). Heatmap showing differentially expressed genes (DEGs) between old (28–29 months) Iba1+ and Iba1− microglia (b). Venn diagram showing overlapping and unique DEGs, between young (2–3 months) and old (28–29 months) Iba1+ and, Iba1− microglia (c). Uniform manifold approximation and projection (UMAP) plot showing distribution of Iba+ and Iba1− microglia between the age groups (d). Quantification of Iba1− microglia in young and old mice (e), quantification and representative (overview and orthogonal projection) images of IBA1−/CX3C motif chemokine receptor 1 (CX3CR1)+ cells in SVZ of young (3–4 months) and old (17–19 months) mice (f). Data presented as % of IBA1− microglia out of total microglia (e) and % of IBA1−/CX3CR1+ of CX3CR1+ cells (f), $n = 3$ (e), $n = 4$ (young) $n = 5$ (aged) (f). Scale bar = 50 μm (overview), 20 μm (orthogonal projection).

we next decided to investigate overall transcriptional changes in aged SVZ microglia.

Microglia in the SVZ drastically change their transcriptional profile with age (Dulken et al., 2019). We used the previously processed data to study potential implications that those changes could have on neurogenesis. Unbiased clustering of SVZ cells divided microglia in two clear cell states (Figure 3a). Interestingly, one state, cluster 4, was dominated by microglia from young mice (Figure 3b), which we refer to as young-like SVZ microglia, whereas cluster 3 was mainly found in aged mice (Figure 3c), further on referred to as aged-like SVZ microglia.

Strikingly, when analyzing this SVZ-unique set of DEGs, we noticed many genes that have been linked to an alternative activation state of microglia, found in models of Alzheimer's disease, known as DAM (Keren-Shaul et al., 2017).

We used DEGs previously identified in DAM from whole brain (Keren-Shaul et al., 2017) and compared them with DEGs, we identified between aged- and young-like SVZ microglia. Interestingly, aged-like SVZ microglia shared a majority of DEGs with DAM (Figure 3d), whereas aged microglia from the whole brain shared only a minority of DEGs with DAM (Figure 3e).

Next, we analyzed all genes, up- and downregulated in DAM and compared expression to SVZ microglia. Strikingly, we observed a clear DAM pattern in aged-like SVZ microglia (Figure 3f,g). In addition, in SVZ microglia, we highlighted the expression of specific genes common with DAM, the upregulation of lipid metabolism and phagosome genes (*Apoe*, *Cst7*, *Lyz2*) (Figure 3h–j), and the downregulation of purinergic receptor gene (*P2ry12*) (Figure 3k). Interestingly, P2ry12, which is linked to phagocytosis (Blume et al., 2020; Haynes et al., 2006), is downregulated in adult SVZ microglia compared to microglia found in other regions (Ribeiro Xavier et al., 2015) and, as shown here, further downregulated in aged-like SVZ microglia compared to young like (Figure 3l).

Finally, we also found that SVZ microglia have a unique aging pattern, independent of DAM, by comparing DEGs between young and old microglia from whole brain and SVZ (Figure 3 m). Among the DEGs, we identified several genes linked to negative regulation of neuronal death (Figure 3n) and negative regulation of extrinsic apoptotic signaling (Figure 3o), many of which were upregulated in aged-like SVZ microglia.

Taken together, our findings indicate that SVZ microglia acquire a unique DAM-like neuroprotective gene expression profile in the aged brain.

3.3 | Heterozygosity of *CX3CR1* increases neurogenesis in the aged SVZ

CX3CR1 is not only a highly specific microglia marker in the murine brain (Ransohoff, 2007) but has also been suggested to support neurogenesis in the hippocampus (Bachstetter et al., 2011; Rogers et al., 2011). However, the role of CX3CR1 has not been well studied in the SVZ or during aging.

Interestingly, we detected a decrease of Cx3cr1 mRNA expression in aged-like SVZ microglia (Figure 4a,b), but not in overall brain microglia. Together with previous studies describing a role of CX3CR1 in DG neurogenesis (Bachstetter et al., 2011), this indicates that CX3CR1 might play a role in regulating SVZ neurogenesis during aging.

To analyze the effect of reduced CX3CR1 levels on SVZ neurogenesis, we compared neuroblast density and proliferation in the SVZ between wild-type (WT) and *Cx3cr1*$^{+/-}$, which display reduced expression of *Cx3cr1* in microglia (Hickman et al., 2019), in young and old mice. We performed immunohistochemistry on coronal brain sections, containing SVZ, for the neuroblast marker, DCX, and the proliferation marker, MCM2.

We first quantified the number of DCX positive neuroblasts or MCM2 positive proliferating cells per mm^2 of SVZ in each age group, as well as DCX/MCM2 double positive proliferating neuroblasts in the DCX+ neuroblast population. In contrast to what have been shown in the DG (Bachstetter et al., 2011), we found no differences between young WT and *Cx3cr1*$^{+/-}$ animals (Figure 4c–e). However, we found a significant increase in *DCX*+ (Figure 4f) *and MCM2*+ (Figure 4g) cell density in aged *Cx3cr1*$^{+/-}$ as compared to WT SVZ, indicating an increase in neuroblast density and overall proliferation in the aged SVZ.

In the same mice, we also quantified MCM2 and DCX positive cells in sections from the DG. We did not find any significant difference in proliferation or neuroblast numbers in the young DG. However, in aged *Cx3cr1*$^{+/-}$ mice, we detected a decrease in a number of DCX+ neuroblasts, whereas proliferating MCM2+ cells were unchanged (Figure S1a–d).

To investigate whether the changes in overall SVZ proliferation was due to increased neuroblast proliferation, we compared the ratio of double positive, DCX+/MCM2+ cells to the total number of DCX+ cells among different groups. Strikingly, we observed a significant increase in DCX/MCM2 double positive cells in aged *Cx3cr1*$^{+/-}$ as compared to aged WT mice (Figure 4h).

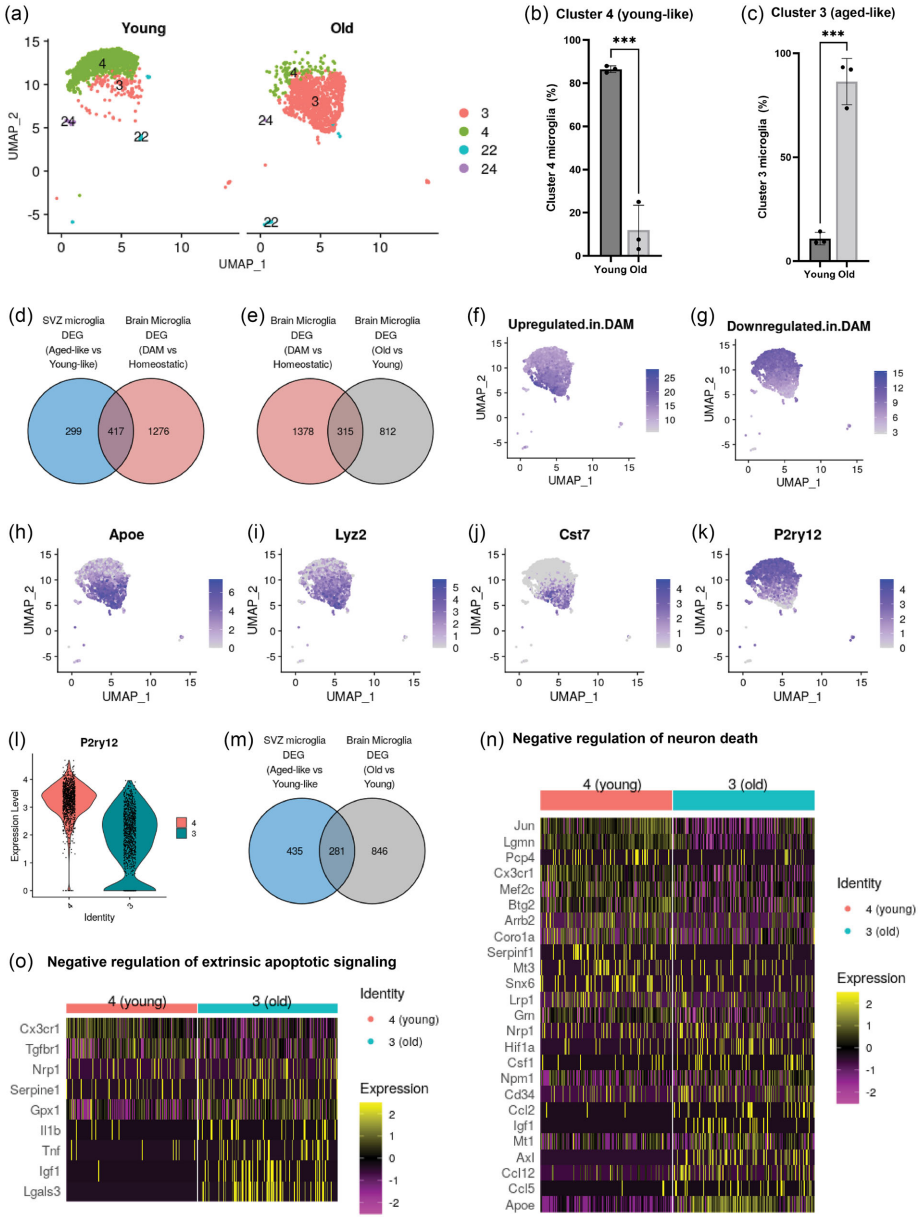

FIGURE 3 **Microglia in the aged murine subventricular zone (SVZ) acquire a unique neuroprotective expression profile**. Uniform manifold approximation and projection (UMAP) plot showing distribution of SVZ microglial states between age groups (a). Quantification of cluster 4 (young-like) microglia in SVZ from young (2–3 months) and old (28–29 months) mice (b). Quantification of cluster 3 (aged-like) microglia in young and old mice (c). Venn diagram showing overlapping and unique differentially expressed genes (DEGs), between SVZ microglia (aged-like vs. young-like) and brain microglia (disease-associated microglia [DAM] vs. homeostatic) (d). Venn diagram showing overlapping and unique DEGs between brain microglia (young (2–3 months) vs. old (21–22 months)) and brain microglia (DAM vs. homeostatic) (e), uniform manifold

(Continues)

FIGURE 3 (Continued)
approximation and projection (UMAP) plot of SVZ microglia showing combined expression of genes upregulated in DAM (f), combined expression of genes downregulated in DAM (g), expression of example genes upregulated in DAM, Apoe (h), Lyz2 (i), and Cst7 (j), and expression of example genes downregulated in DAM, P2ry12 (k). Violin plot of P2ry12 expression in cluster 4 (young-like) and cluster 3 (aged-like) SVZ microglia (l). Venn diagram showing overlapping and unique DEGs between SVZ microglia (aged-like vs. young-like) and brain microglia (young vs. old) (m). Heatmap of DEGs between cluster 4 (young-like) and cluster 3 (aged-like) with the gene ontology (GO) tag "negative regulation of neuron death" (n). Heatmap of DEGs between cluster 4 (young-like) and cluster 3 (old-like) with the GO-tag "negative regulation of extrinsic apoptotic signaling" (o). Data presented as cells per total microglia (b and c), $n = 3$ (b and c).

Taken together, these findings show that the heterozygosity of CX3CR1 increases SVZ proliferation, neuroblast density, and neuroblast proliferation specifically in aged mice, while having no or opposite effect in the DG.

4 | DISCUSSION

Here we have performed a detailed transcriptional analysis of the SVZ neurogenic niche microglia during aging. We show that Iba1+ and Iba1− microglia subpopulations have very similar transcriptional profiles in the aged SVZ and adopt a DAM-like neuroprotective phenotype. Furthermore, we show that Cx3cr1 is downregulated during aging and that Cx3cr1 haploinsufficiency increases neurogenesis specifically in the aged SVZ.

SVZ microglia have unique properties, contain subpopulations, and have been suggested to support neurogenesis (Ribeiro Xavier et al., 2015). We asked whether one of these subpopulations, the Iba1− microglia, changes with age and potentially influences the declining neurogenesis. Surprisingly, we detected no change in number and remarkably little transcriptional difference of this population with age. These results imply that the Iba1− population is not actively involved in regulating neurogenesis in the aged brain. However, we cannot exclude that other minor microglia subpopulations or other immune cells exist within the aged SVZ that could exhibit more pronounced transcriptional change and affect neurogenesis during aging. Interestingly, a recent study described the presence of T-cells with detrimental effect on neurogenesis in the aged SVZ (Dulken et al., 2019), highlighting the need for future studies detailing the immune cells present and their interaction in aged neurogenic niches. So far most studies have examined mice at young and aged time points. It will be important to assess additional intermediate time points during aging as one cannot exclude that changes observed occur very early on and not necessarily a consequence of old age.

Our analysis suggests that SVZ microglia undergo distinct transcriptional changes during aging as compared to microglia in the rest of the brain, and largely resemble DAM. It has been reported that the aged brain harbors 3% DAM (Keren-Shaul et al., 2017), which together with our analysis indicates that the aged SVZ accounts for a significant part of DAM in the aged brain. New aging datasets with separated brain region would help to clarify regional distribution of DAM. Several studies show that microglia can acquire different types of activation depending on stimuli, DAM being one of many. In addition, it has previously been shown that aged microglia are primed for activation (Niraula et al., 2017), and it has been suggested that age-related dysregulation may interfere with microglial transition to DAM (Deczkowska et al., 2017; Keren-Shaul et al., 2017). The unique transition observed in aged SVZ microglia could be priming toward or a fully activated state. Gradient expression between major cell states allows for parallel conditions. Another possibility is that SVZ microglia during aging initiate activation toward DAM but either loose its path or stop before fully transforming into DAM.

Apoptotic immature neurons in the hippocampus decrease with age and are cleared by phagocytic microglia (Sierra et al., 2010; Sierra et al., 2013). Among genes downregulated in DAM, we found P2ry12 to be downregulated in aged-like SVZ microglia but not in aged microglia from the rest of the brain. P2ry12 is a homeostatic gene (Deczkowska et al., 2018), but important for phagocytosis (Blume et al., 2020). Interestingly, reduced phagocytic activity by removing P2ry12 resulted in reduced neurogenesis (Diaz-Aparicio et al., 2020). Reduced phagocytic activity has been reported in the aged brain, including the hippocampus (Safaiyan et al., 2016), where it is thought to be a result of reduced number of apoptotic immature neurons in the neurogenic niche (Sierra et al., 2010). DAM, on the other hand, have increased phagocytic activity to clear protein aggregates (Deczkowska et al., 2018), thus acting in a neuroprotective manner. Microglia not only react to apoptotic cells but can also regulate apoptosis through its secretome to affect the survival of newly formed neurons (Guadagno et al., 2013). We found several DEGs in aged-like SVZ microglia with potential to reduce apoptosis of immature neurons and act neuroprotective. However, to what extent, this population really is neuroprotective remains to be established.

NSCs exist in a quiescent state before they get activated to generate new neurons (Urban et al., 2019). During aging, a subset of quiescent neural stem cells (qNSCs) exhibits fewer and defective lysosomes, which result in reduced autophagy and accumulation of protein aggregates (Leeman et al., 2018). In addition, aged qNSCs reduce their ability to activate

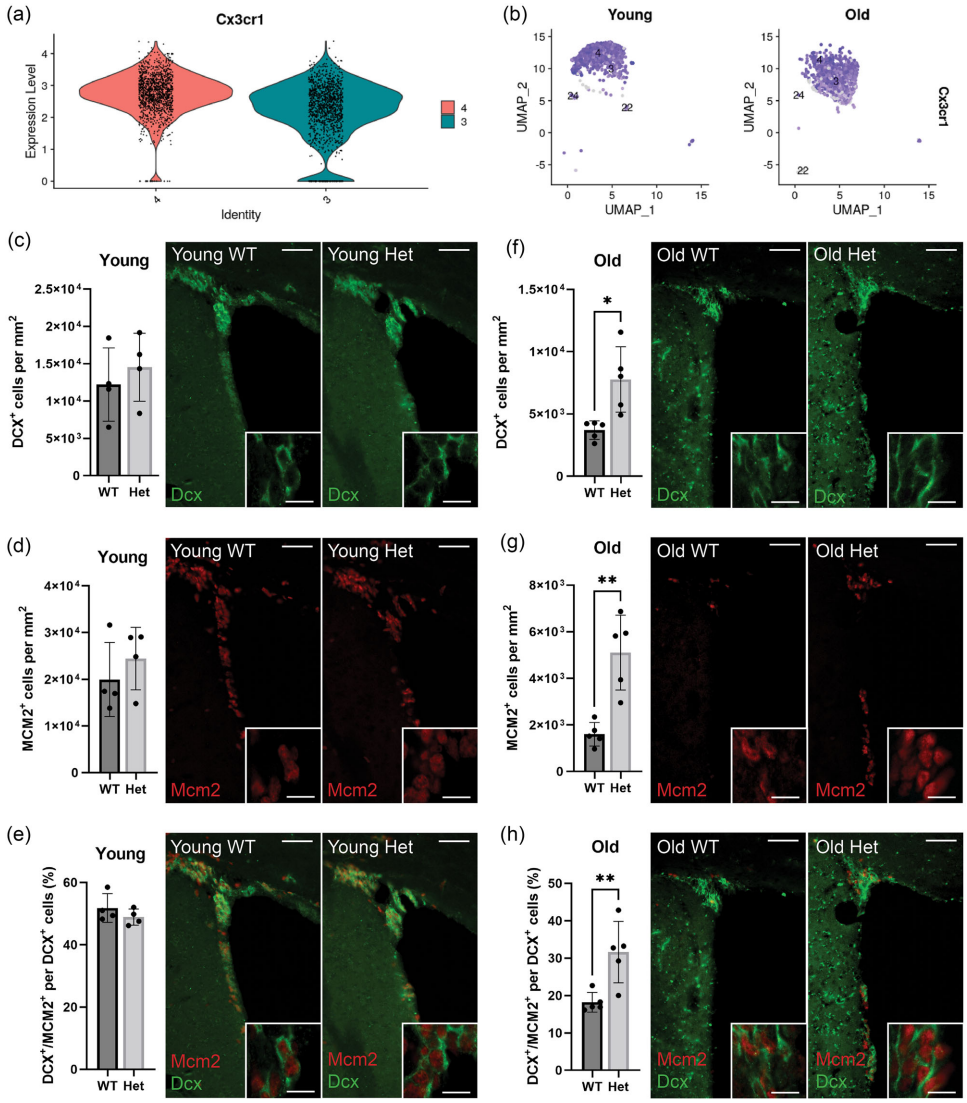

FIGURE 4 **Decreased expression of CX3C motif chemokine receptor 1 (Cx3cr1) leads to increased proliferation and neuroblast density in the aged subventricular zone (SVZ)**. Violin plot of Cx3cr1 expression in cluster 4 (young-like) and cluster 3 (aged-like) SVZ microglia (a). Uniform manifold approximation and projection (UMAP) plot of SVZ microglia showing expression of Cx3cr1 (b). Quantification and representative (overview and high magnification) images of doublecortin (DCX+) (c), minichromosome maintenance 2 (MCM2+) (d), and DCX+/MCM2+ (e) cells, in the SVZ of young (3–4 months old) wild-type (WT) and Cx3cr1$^{+/-}$ mice. Quantification and representative (overview and high magnification) images of DCX+ (f), MCM2+ (g), and DCX+/MCM2+ (h) cells, in the SVZ of old (17–19 months old) WT and Cx3cr1$^{+/-}$ mice. Data presented as mean cell density (cells/mm^2) per 30 μm section ± SEM; $n = 4$ (b–d), $n = 5$ (e–g). Scale bar = 50 μm (overview), 10 μm (high magnification).

(Enwere et al., 2004), which can be countered by clearing protein aggregates through increased lysosomal function and enhanced autophagy (Leeman et al., 2018). Microglia clear extracellular protein aggregates released by other cells (Plaza-Zabala et al., 2017; Sole-Domenech et al., 2016) where DAM play an important neuroprotective role in neurodegenerative disease. It is tempting to speculate that DAM in the aged SVZ regulate neurogenesis by clearing aggregated proteins from stem and progenitor cells. However, whether microglial interaction contributes to lysosomal function in neighboring qNSCs and if DAM-like microglia have a similar important role in the neurogenic niches as in neurodegenerative disorders remains to be established.

We show here for the first time that haploinsufficiency of Cx3cr1 results in increased proliferation and neuroblast density in the aged, but not the young mouse SVZ. Interestingly, we found *Cx3cr1* expression to be uniquely decreased in aged-like SVZ microglia.

These results are counterintuitive, as the reduced *Cx3cr1* expression observed in old microglia should then increase neurogenesis. One possible explanation is that aging microglia adapt their transcriptional profile to mitigate the declining neurogenesis but cannot fully prevent neurogenic decline.

It is important to note that, similar to our transcriptomic analysis, we cannot exclude that differences observed in aged mice might initiate early in life.

Many immune cells outside of the brain express Cx3cr1 (Lee et al., 2018) and could potentially regulate neurogenesis through cytokines that pass the blood–brain barrier (Gutierrez et al., 1993). In addition, monocytes and macrophages, which express the receptor, infiltrate the aged brain and have the potential to modulate neurogenesis (Finger et al., 2021). However, microglia is by far the largest population that expresses Cx3cr1 in the brain and in the neurogenic niches. It is therefore most likely that the changes observed are predominantly mediated through microglia and that different microglial populations in SVZ and DG affect neurogenesis differently in the two niches.

Previous studies show decreased neurogenesis in the adult and aged CX3CR1-deficient DG (Bachstetter et al., 2011; Rogers et al., 2011), a process seemingly independent of the ligand, CX3CL1 (Sellner et al., 2016). Specifically, increased proliferation and decreased number of neuroblasts were observed in CX3CR1-deficient mice. In contrast, our experiments did not reveal any changes in the young SVZ or DG but showed an increase in both proliferation and neuroblast numbers in the aged SVZ. There are several potential explanations for this discrepancy. In our study, we examined mice heterozygous for Cx3cr1, whereas previous studies have used mice with complete knockout of the receptor. A more likely explanation, however, is that NSCs, microglia, and other cell types are very different in the SVZ and DG neurogenic niches and could therefore adapt differently to CX3CR1 deficiency. In support of this notion, we mainly observed differences in the SVZ and not in the DG. The decreased number of neuroblasts observed in the aged DG agrees with previous studies and further strengthens the idea that the two neurogenic regions are regulated differently. However, in general, both number of proliferative cells and neuroblast numbers are much lower in the aged DG as compared to aged SVZ, making analysis challenging and calling for multi-time point studies.

Enhancing CX3CR1/CX3CL1 interactions has been suggested as a therapeutic approach to slow age-related neurodegeneration (Bachstetter et al., 2011). In light of our findings, it is important to note that altering CX3CR1/CX3CL1 signaling in patients might have different impacts on neurogenesis at different ages and in different regions of the brain.

5 | CONCLUSION

Our study shows that aged SVZ microglia acquire an expression profile similar to DAM and identifies CX3CR1 as a novel, age- and niche-specific, regulator of adult neurogenesis. Our findings suggest that SVZ microglia adapt their gene expression to counteract the neurogenic decline in aging. This work adds to the understanding of the dynamic role of microglia in regulating adult neurogenesis and highlights the need for further longitudinal studies to disentangle the complex interaction between microglia and NSCs during aging.

AUTHOR CONTRIBUTIONS

Jonas Fritze: Conceptualization; data curation: software; investigation; formal analysis; validation; writing—original draft; visualization; writing—review and editing; supervision; project administration. **Chandramouli Muralidharan**: Conceptualization; data curation; software; investigation; formal analysis; validation; writing—original draft; writing—review and editing. **Eleanor Stamp**: Investigation; formal analysis; validation; writing—review and editing. **Henrik Ahlenius**: Conceptualization; investigation; resources; supervision; project administration; funding acquisition; writing—review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflicts of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available from the corresponding authors upon reasonable request. Raw sequencing data used in this study were derived from the National Center for Biotechnology Information (NCBI) under BioProject with reference number PRJNA450425 at https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA450425 and under Gene Expression Omnibus (GEO) with reference number GSE98969 and GSE129788 at https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE98969 and https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE129788, respectively.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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Single-cell RNA sequencing of aging neural progenitors reveals loss of excitatory neuron potential and a population with transcriptional immune response

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In the adult murine brain, neural stem cells (NSCs) can be found in two main niches: the dentate gyrus (DG) and the subventricular zone (SVZ). In the DG, NSCs produce intermediate progenitors (IPs) that differentiate into excitatory neurons, while progenitors in the SVZ migrate to the olfactory bulb (OB), where they mainly differentiate into inhibitory interneurons. Neurogenesis, the process of generating new neurons, persists throughout life but decreases dramatically with aging, concomitantly with increased inflammation. Although many cell types, including microglia, undergo significant transcriptional changes, few such changes have been detected in neural progenitors. Furthermore, transcriptional profiles in progenitors from different neurogenic regions have not been compared on a single-cell level, and little is known about how they are affected by aging-related inflammation. We have generated a single cell RNA sequencing dataset enriched for IPs, which revealed that most aged neural progenitors only acquire minor transcriptional changes. However, progenitors set to become excitatory neurons decrease faster than others. In addition, a population in the aged SVZ, not detected in the OB, acquired major transcriptional activation related to immune responses. This suggests that differences in age related neurogenic decline between regions is not due to tissue differences but rather cell type specific intrinsic transcriptional programs, and that subset of neuroblasts in the SVZ react strongly to age related inflammatory cues.

KEYWORDS

neurogenesis, aging, intermediate progenitors, neuroblasts, immune response, dentate gyrus, subventricular zone, excitatory

1 Introduction

In the adult murine brain, quiescent neural stem cells (NSCs) can be found in two main niches: the subgranular zone (SGZ) of the dentate gyrus (DG), which lies in the hippocampus, and the subventricular zone (SVZ) lining the lateral ventricles (Jurkowski et al., 2020). Upon activation, neural stem cells (NSCs) self-renew and/or differentiate to produce intermediate progenitors (IPs), called type-2 cells in the SGZ and transit-amplifying progenitors (TAPs) in the SVZ, which have the potential to develop into

various cell types of the brain (Bonaguidi et al., 2016). To generate neurons, early IPs develop into neuroblasts, another IP state, which differentiate into immature neurons before transforming into fully mature neurons. In the DG, neuroblasts migrate to the granule cell layer where they primarily differentiate into excitatory granule neurons, while neuroblasts in the SVZ migrate via the rostral migratory stream to the olfactory bulb (OB) where they mainly mature into inhibitory interneurons (Lledo et al., 2006).

Neurogenesis, the process by which NSCs generate neurons, persists throughout life but decreases drastically with age. The NSC pools become smaller and lose functionality with aging, leading to a decline in the proliferation of IPs, which results in fewer newly formed neurons (Katsimpardi and Lledo, 2018). Consequently, neurogenesis-dependent functions, such as olfactory discrimination and pattern separation, decline with aging (Alonso et al., 2006; Breton-Provencher et al., 2009; Lazarini et al., 2009; Moreno et al., 2009; Johnston et al., 2016; Dillon et al., 2017). Simultaneously, brain inflammation increases with microglia, which are the immune cells of the brain, reacting to aging (Wendimu and Hooks, 2022) and peripheral immune cells infiltrating the central nervous system (Dulken et al., 2019).

Single-cell sequencing has been used to study different cell types during aging. Many cell types, including microglia, undergo dramatic transcriptional changes; however, few such changes have been detected in IPs, likely because their relatively low numbers (Ximerakis et al., 2019) make their analysis challenging. While NSCs have been studied extensively in the context of aging, IPs from different regions have not been compared, and little is known about how inflammation and aging affect the transcriptional integrity of neuroblasts and the potential for new neurons to integrate in the aged brain.

We generated a single-cell RNA sequencing dataset enriched for IPs from the SVZ, OB, and DG of adult, middle-aged, and aged mice. The dataset revealed that most IPs acquire only minor transcriptional changes. Additionally, it showed that neuroblasts set to become excitatory neurons decrease faster than other neuroblast populations, and a neuroblast population in the aged SVZ, not found in the OB, acquires major transcriptional activation related to immune responses.

2 Materials and methods

2.1 Animals

All experimental procedures were approved by the Malmö–Lund Ethical Committee for the use of laboratory animals and were conducted following the European Union directive on animal rights. Mice expressing a green fluorescent protein (GFP) under the Dcx promoter (Dcx-GFP) (Couillard-Despres et al., 2006), which is highly active in neuroblasts, were bred and housed in the animal facility connected to the Lund University Biomedical Center under sterile conditions in individually ventilated cages (IVCs) at 22°C, with 40%−60% humidity and a 12-h light/dark cycle with *ab libitum* access to food and water. Adult (3–4 months), middle-aged (12–16 months), and aged (18–24 months) mice were used, with male and female mice equally distributed between groups. Tissues from four to five mice were pooled for each age group.

2.2 Brain dissection and tissue dissociation

Heterozygote Dcx-GFP mice were euthanized by cervical dislocation, and their brains were kept in an ice-cold L-15 medium (Invitrogen: Waltham, Massachusetts, United States), as previously described (Ahlenius and Kokaia, 2010). To facilitate the comparison of neurogenic niches, the OBs were isolated and the SVZs and DGs were dissected from 1-mm sections separately. Tissue from 4-5 mice were pooled in ice-cold L-15 medium separately for each region (from the same group of animals) and dissociated into a single -cell suspension using the Adult Brain Dissociation Kit, mouse and rat (Miltenyi Biotech: Bergisch Gladbach, North Rhine-Westphalia, Germany, 130-107-677) following the manufacturer's guidelines. The cells were then purified through a two-step gradient as previously described (Ahlenius and Kokaia, 2010) and resuspended in fluorescence-activated cell sorting (FACS) medium [4% Bovine serum albumin (BSA) (W/V), 2% 2M 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) (V/V), and 0.1% Sodium azide (V/V) in Hanks' balanced salt solution (HBSS) (BSA, HEPES and HBSS: Thermo fisher; Waltham, Massachusetts, United States)].

2.3 Fluorescence-activated cell sorting

To enrich for neural progenitors, dissociated cells from Dcx-GFP mice were subjected to fluorescence-activated cell sorting (FACS; Supplementary Figure 1a) for separation based on their endogenous GFP expression, and cell viability was assessed with propidium iodide using FACSARIAII/III (BD Biosciences: Franklin Lakes, New Jersey, United States). The sorted cells were collected into Phosphate-buffered saline (PBS) with 2% Fetal bovine serum (FBS) for single-cell sequencing library preparation on the same day. Alternatively, these cells were collected into QIAzol lysis reagent (Qiagen: Venlo, Netherlands (corporate) Hilden, Germany (operational)) and stored at −80°C for bulk sequencing.

2.4 RNA extraction

For bulk RNA sequencing, total RNA was isolated from the sorted GFP+ cells using the miRNeasy Micro Kit (Qiagen) according to the manufacturer's instructions. Briefly, the samples frozen in the QIAzol lysis reagent were brought to room temperature and homogenized by pipetting, and the RNA was then separated with a chloroform phase gradient. During RNA purification, on-column DNase digestion (Qiagen) was performed, and the final RNA was eluted into 14 μl of H_2O. Concentrations were measured using a bioanalyzer (Agilent: Santa Clara, California, United States) and loaded using the RNA 6000 Pico Kit (Agilent).

2.5 Library preparation and sequencing

For single-cell RNA sequencing, libraries were generated from FACS-sorted cells expressing endogenous GFP (Dcx-GFP) for each experimental group separately using a Chromium system (10×

Genomics: Pleasanton, California, United States), according to the manufacturer's guidelines [Single Cell 3′ Reagent Kits v2 User Guide (CG00052)], with 12–13 cDNA amplification cycles and 14 sample index cycles. The libraries generated from 2,100 aged DG cells, 3,000 aged OB cells, and 5,000 cells for all other samples were sequenced on a NextSeq 500 System (Illumina: San Diego, California, United States) using NextSeq 500/550 High Output v2 Kits (Illumina). Each sample contained cells from four to five mice. The cells from different regions but of the same age group were obtained from the same group of animals.

Bulk RNA sequencing libraries were prepared using the SMARTer Stranded Total RNA-Seq Kit v2, Pico Input Mammalian (Takara Bio USA, Inc.) according to the manufacturer's user manual (063017). Furthermore, 2 ng of total RNA was used from each sample with 4-min fragmentation time and 14 PCR cycles for amplification of the final library in a Mastercycler X50s (Eppendorf: Hamburg, Germany). The libraries were sequenced on a NextSeq 500 System (Illumina) using NextSeq 500/550 High Output v2.5 Kits (Illumina). The sequencing was performed at the Center for Translational Genomics, Lund University, and Clinical Genomics Lund, SciLifeLab. Each biological sample ($n = 5$) contained cells from four to five mice for the bulk data analysis. The cells from all different regions were collected from every animal included in the study.

2.6 Raw data processing and quality control

Raw data from single-cell RNA sequencing were processed through the Cell Ranger pipeline to generate count matrices, which were then loaded into the R computational environment (4.1.2) using read10xCounts (DropletUtils) and merged into a single-cell experiment object (i.e., SingleCellExperiment). The counts were normalized using logNormCounts (Scater) with size factors based on quickCluster and computeSumFactors (Scran). Then, the logcounts were extracted as dgCMatrices and merged into a Seurat object using CreateSeuratObject (Seurat). The low-quality cells with fewer than 75 or more than 4,000 detected genes (features), more than 1.5% mitochondrial genes, or more than 15% ribosomal genes were discarded (Supplementary Figure 1b). A very low number of cells expressing high levels of Cx3cr1 and Iba1, which are markers for microglia, were also discarded.

For bulk sequencing, FASTQ files were organized using bcl2fastq2 with default settings and without warnings from FastQC (Supplementary Figures 2a–c). Reads were aligned to the mouse reference genome (GRCm38) from the Ensemble database using HISAT2 (Supplementary Figures 2d, e). Quality control of the aligned data, including base distribution and insert size checks, was performed using Picard (Supplementary Figure 2a). Assembly of the alignments into full transcripts and quantification of expression levels were performed using StringTie.

2.7 Clustering and cell type assignment

The data analysis was performed in the R computational environment (4.1.2). All genes were scaled with ScaleData (Seurat) to accurately compare the expression between cells. The 2,000 most highly variable genes (HVGs; Supplementary Figure 1c) were identified using the variance stabilizing transformation (VST) method via FindVariableFeatures (Seurat), which were then used for the principal component analysis. The first 20 principle components with the highest standard deviation were selected manually from an elbow plot and used for Louvain clustering (Seurat) with a k parameter of 10 and a resolution of 1 (Figure 1A). Clusters were visualized with uniform manifold approximation and projection (UMAP), initially annotated using singleR (1.8.1) based on a reference mouse brain dataset from Celldex (Benayoun et al., 2019) (Figure 1B), and further adjusted manually to commonly used markers (i.e., Glial fibrillary acidic protein (Gfap) for astroglial and Neural stem cells (NSCs), Achaete-scute family bHLH transcription factor 1 (Ascl1) for NSCs and early progenitors, Doublecortin (Dcx) for neuroblasts, Synaptotagmin 1 (Syt1) for neurons, Platelet derived growth factor receptor alpha (Pdgfra) for oligodendrocyte precursors, and Mbp for mature oligodendrocytes; Figures 1C–H). The clusters assigned as oligodendrocytes were discarded, along with the clusters where more than 40% of the population expressed Gfap and <40% expressed Ascl1, which were considered astroglial populations without activated NSCs. DimPlot (Seurat) was used to visualize the clusters, while FeaturePlot (Seurat) was used to illustrate gene expression.

2.8 Differential gene expression analysis and quantifications

For single-cell RNA sequencing data, differential gene expression analysis was performed using findMarkers (Seurat), considering p-values based on Bonferroni correction, using all genes in the dataset; the adjusted p-values (p_val_adj) of <0.05 are considered statistically significant. ComplexUpset was used for upset plots, Ggplot (ggplot2) for boxplots and extreme point plots, and DoHeatmap (Seruat) for heatmaps. Gene ontology (GO) terms were identified for differentially expressed genes (DEGs), with the adjusted p-values refers to how the DEGs were chosen, while other settings refers to the enrichGO function from ClusterProfiler (4.2.2), with parameters set to minGSSize 1, maxGSSize 500, and both qvalue Cutoff and pvalueCutoff were set to 1. GraphPad Prism v7 was used to visualize population size estimation data.

For bulk RNA sequencing data, DESeq2 was used for differential gene expression (DGE) analysis, which compares adult samples to a combined group of middle-aged and aged samples. Heatmaps were generated using pheatmap, color pallets were created using RColorBrewer, and bar graphs were created using GraphPad Prism v7. Inflammatory response genes can considerably vary due to infection or other external stimuli; therefore, to identify outliers, two independent statistical tests were conducted: Cooks' distance (Supplementary Figures 3a, b) and Grubbs' test (Supplementary Table 1). One adult sample was identified as an outlier for inflammation-related genes in both tests and was not included in the DGE analysis for inflammatory response genes. The DGE analysis included four or five biological replicates, each pooled with the RNA of four to five adult mice. Furthermore, 10 middle-aged and aged mice were included in the DGE analysis.

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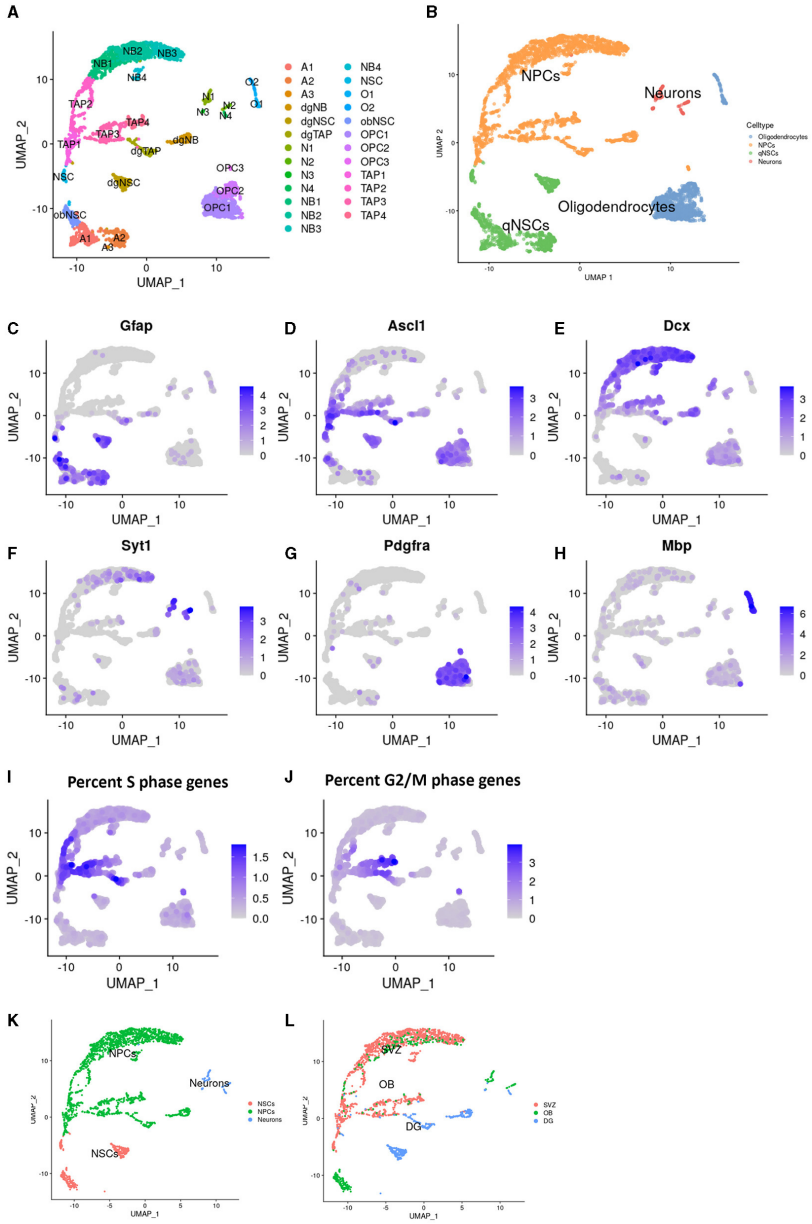

FIGURE 1
Identification of populations in Dcx+ cells sorted from neurogenic niches. Uniform manifold approximation and projection (UMAP) plots showing cluster names **(A)** and cell type annotation **(B)**. Visualization of common markers, in the UMAP, for Astrocytes and NSCs; Gfap **(C)**, TAPs; ASCl1 **(D)**, Neuroblasts; Dcx **(E)**, Neurons; Syt1 **(F)**, OPCs; Pdgfra **(G)**, and Oligodendrocytes; Mbp **(H)**. Percentage of genes prominently expressed in the s-phase **(I)** and g2- and m- phase of the cell cycle **(J)**. Cell type annotation after cell filtering **(K)** and regional identity **(L)** in the UMAP.

Cell numbers were normalized to OPCs since it is the population in our data set most stable during aging and present in large enough quantity.

3 Results

3.1 Aged neuroblasts display minor but cell state dynamic transcriptional changes

NSCs are fewer in number and less active in the aged brain compared to the adult mammalian brain. This decline leads to the production of fewer IPs and, ultimately, fewer new neurons added to the brain during aging (Katsimpardi and Lledo, 2018). Although recent research has revealed age-related changes in stem cells, little is known about how IPs such as neuroblasts are affected by aging.

To study the effect of aging on IPs, including TAPs and neuroblasts, in the two major neurogenic niches, we dissected the SVZ, OB, and DG from 3-, 14-, and 24-month-old Dcx-GFP mice. The GFP+ cells were isolated using FACS and single-cell RNA sequencing was performed using 10× and Illumina chemistry. During data processing, we performed unbiased clustering of cells from all ages and regions, which were digitally pooled together (Figure 1A). Clusters were annotated computationally based on previous RNA sequencing studies (Figure 1B) and manually verified and further specified using common markers. Cells in clusters with high expression of both Gfap and Ascl1 were annotated as NCSs, with Ascl1 alone as TAPs, Dcx as neuroblasts, Syt1 as mature neurons, Pdgfra as oligodendrocyte progenitors, and Mbp as mature oligodendrocytes (Figures 1C–H). The expression of cell cycle genes (Figures 1I, J) highlighted highly proliferative cells, such as TAPs.

We identified all major common cell types involved in neurogenesis with a strong enrichment of IPs. Interestingly, some clusters split into substates that were not previously described (Figure 1A), indicating that enrichment, by FACS, for neural progenitors aided in revealing their full heterogeneity.

Clusters with cells from neuronal lineages were selected for further downstream analysis (Figure 1K). We identified two differentiation trajectories from NSCs to mature IPs in the SVZ (Supplementary Figure 1d), one leading to IPs with a potential to generate inhibitory neurons and one to excitatory neurons as previously described (Sequerra et al., 2013). We identified a unique NSC population in the OB, as previously reported (Defterali, 2021), initiating a trajectory leading to inhibitory neurons (Supplementary Figure 1e). In the DG, we identified a single trajectory leading to excitatory neurons (Supplementary Figure 1f).

As expected, from previous histological studies (Jurkowski et al., 2020), the cells from the DG distinctly separated from most cells in the SVZ or OB, and the majority of mature cells could be detected only in the OB or DG. Most cell types at various maturity states identified in the SVZ were also detected in the OB, albeit to a lesser extent, and only a few SVZ and OB cells were clustered with DG cells (Figure 1L).

For each cluster where the population size remained consistent with age and there were sufficient cells in each age group to run the analysis, we performed the DEG analysis between adult and aged cells in the SVZ, OB, and DG separately (Figures 2A, B). We found only a few genes that were upregulated or downregulated in most aged cell types in the DG and SVZ and none in the OB. In the SVZ, none of these DEGs were universal for all cell types; while some were shared, others were unique. The panel of DEGs also differed between neighboring transient cell states, although a larger proportion was shared (Figure 2C).

All non-cycling neuroblast clusters (NB1, NB2, and NB3) exhibited very similar gene expression profiles (Figure 2C) and shared DEGs with age. We, therefore, decided to digitally pool them together to achieve a more powerful DEG analysis, revealing several genes involved in axonogenesis, such as Tubb3, Stmn1, and Nr4a2, to be differentially expressed between age groups (Figure 2D). Tubb3 is a major component of axons (Radwitz et al., 2022) and, along with Nr4a2 (Nurr1) and Stmn1, has been shown to promote neurite growth (Heng et al., 2012; Latremoliere et al., 2018; Kwon et al., 2020).

Next, we performed differential gene expression analysis between adult and middle-aged and between middle-aged and aged SVZ clusters. We detected several genes to have an expression maximum in the mid aged group that either dropped to, or below adult levels in the aged group (Figure 2E). This indicates that transcriptional changes occurring during aging are not always monotonic but dynamic. Many of these extreme-point genes coded for ribosomal proteins in the neuroblast clusters, while *Hist1h2ap*, a gene encoding a replication-dependent histone, was prominent in the TAP3 cluster of cycling IPs (Supplementary Figure 1b; Figure 2E).

In summary, aging leads to only a few differentially expressed genes in IPs, with some unique to specific cell types and some shared, but none universal. Despite the small changes, our data indicate that transcriptional changes during aging can be non-monotonic and that aged neuroblasts differentially express genes involved in axon development.

3.2 Excitatory neuron progenitors in the SVZ and DG decrease faster during aging than inhibitory neuron progenitors

NSCs in the adult DG primarily form excitatory neurons, while SVZ neurogenesis mainly produces inhibitory interneurons (Lledo et al., 2006). Having established that DG and SVZ IPs have different gene expression profiles and rarely share aging-related transcriptional changes, we next assessed if aging affects neural progenitor heterogeneity.

To investigate IP heterogeneity during aging, we used clusters generated from digitally pooled samples (Figure 1A) and identified neural progenitors fated for different neuronal subtypes based on previously identified genes. We used Gad1 (Gad67) and St18 [identified here, and known to regulate inhibitory neuron fate (Nunnelly et al., 2022)] to highlight IPs set to become inhibitory interneurons and Eomes (Tbr2), Tbr1 and Slc17a6 (Vglut2) for IPs set to become excitatory neurons. Excitatory markers were clearly expressed in DG IPs in contrast to the majority of SVZ IPs that instead expressed Gad1. However, when separating the two neurogenic regions, we found that a small fraction of SVZ IPs clustered with DG IPs and expressed markers for excitatory

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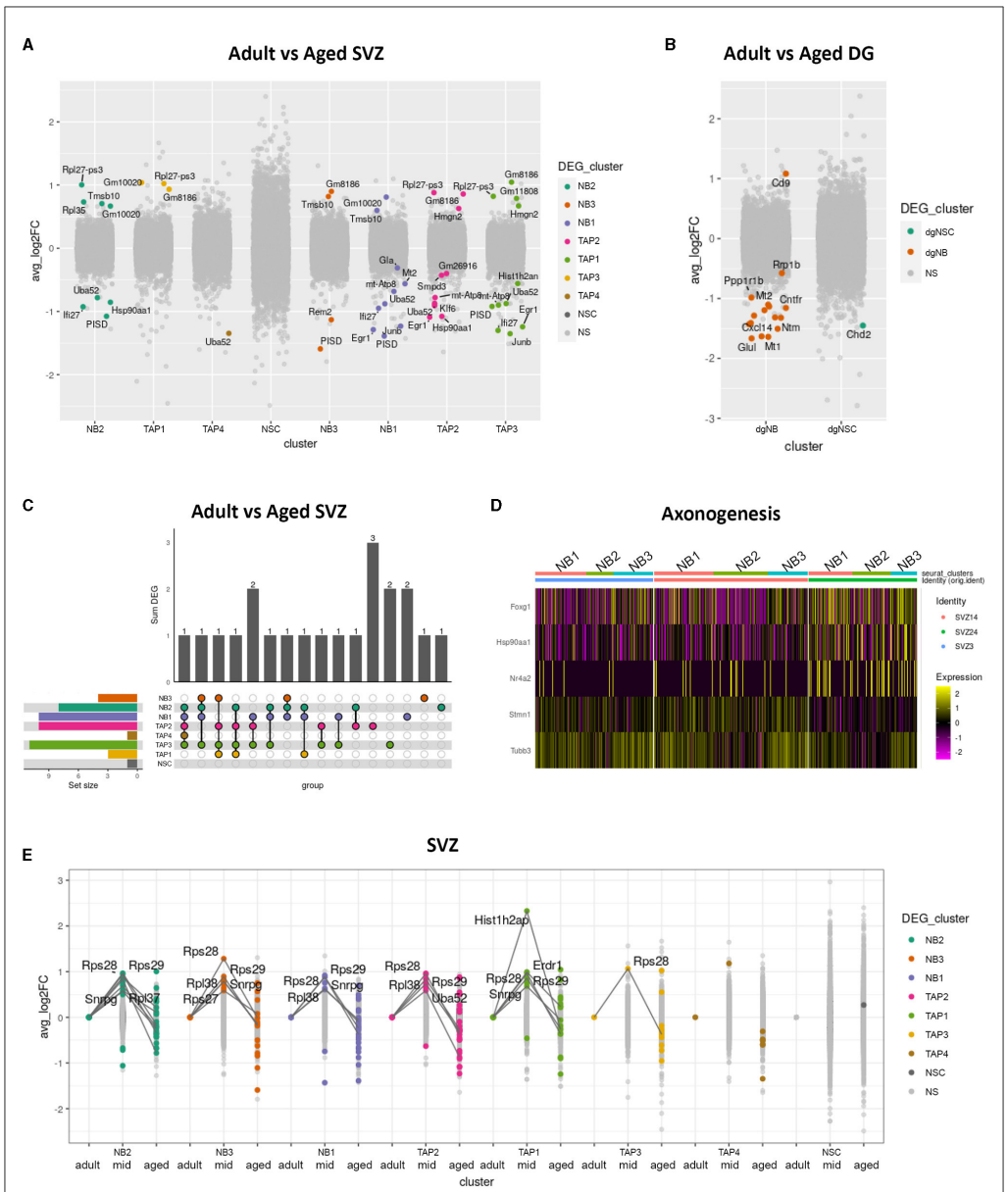

FIGURE 2
Differential gene expression in aged neurogenic niches. Boxplot showing differentially expressed genes ($p\_val\_adj < 0.05$) in color and average log fold changes of all genes between adult and aged clusters of the SVZ **(A)** and the DG **(B)**. Upset plot showing shared and unique differentially expressed genes between adult and aged clusters in the SVZ **(C)**. Heatmap showing differentially expressed genes between adult and aged SVZ clusters NB1, NB2, and NB3, combined for genes with the common gene ontology term "axonogenesis" **(D)**. Extreme point plot where lines highlight genes with expression max or min in the middle-aged group of different clusters in the SVZ and where colored dots indicate differential expression compared to adult **(E)**.

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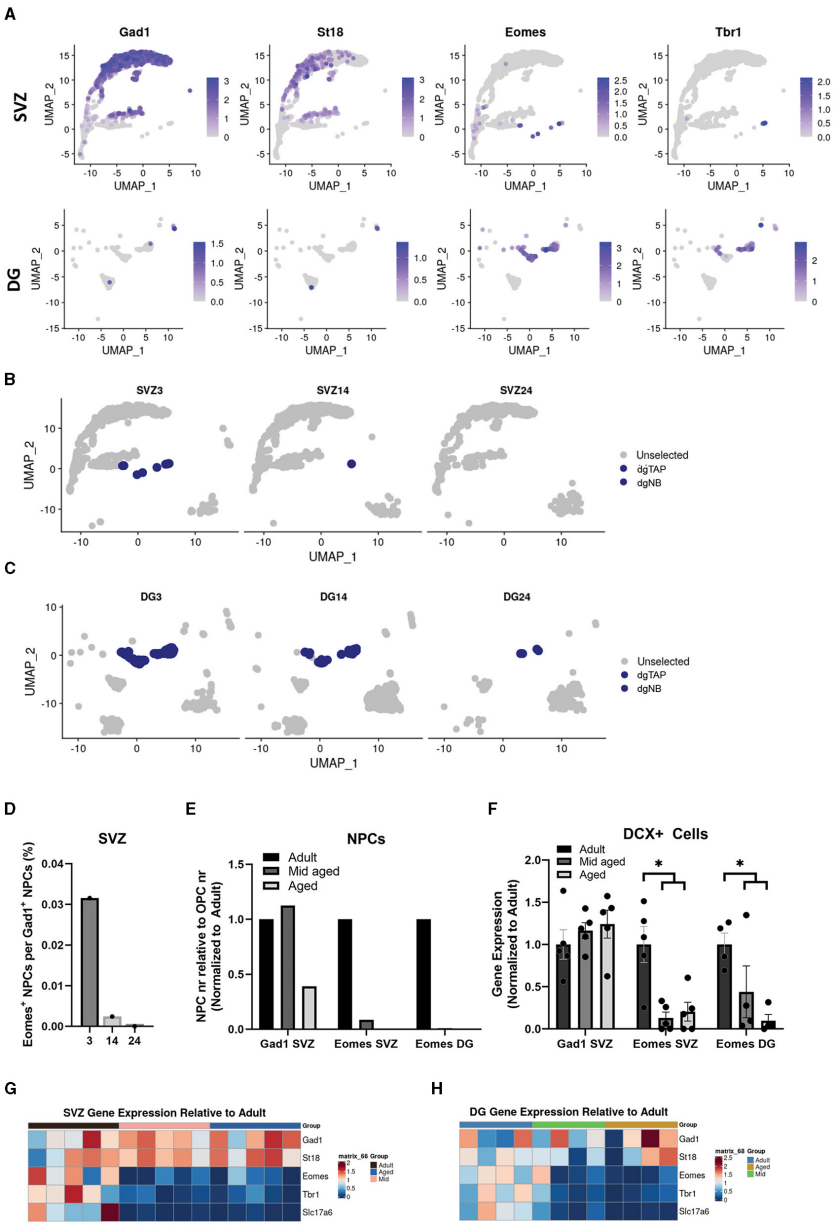

FIGURE 3
Excitatory neuron progenitors in the SVZ and DG decrease faster during aging than inhibitory neuron progenitors. Uniform manifold approximation and projection (UMAP) showing expression of common markers for inhibitory neurons, Gad1 and St18, and for excitatory neurons, Eomes and Tbr1, in the SVZ and DG **(A)**. Cluster dgTAP and dgNB highlighted in different age groups of the SVZ **(B)** and DG **(C)**. Quantification of cells in non-cycling cell populations expressing either Eomes (cluster dgTAP and dgNB) or Gad1 (cluster NB1, NB2, and NB3) in the SVZ, represented as a ratio **(D)**, and in the SVZ and DG represented as relative to OPCs and normalized to adult **(E)**. Gene expression normalized to adult of Gad1 and Eomes in bulk sequencing samples, *Adjusted p-value of < 0.05, with DESeq2, of adult samples compared to a combined group of middle-aged and aged samples **(F)**. Heatmap showing gene expression of common markers for inhibitory neurons, Gad1 and St18, and for excitatory neurons, Eomes, Tbr1, and Slc17a6, from different bulk samples relative to mean adult in the SVZ **(G)** and DG **(H)**.

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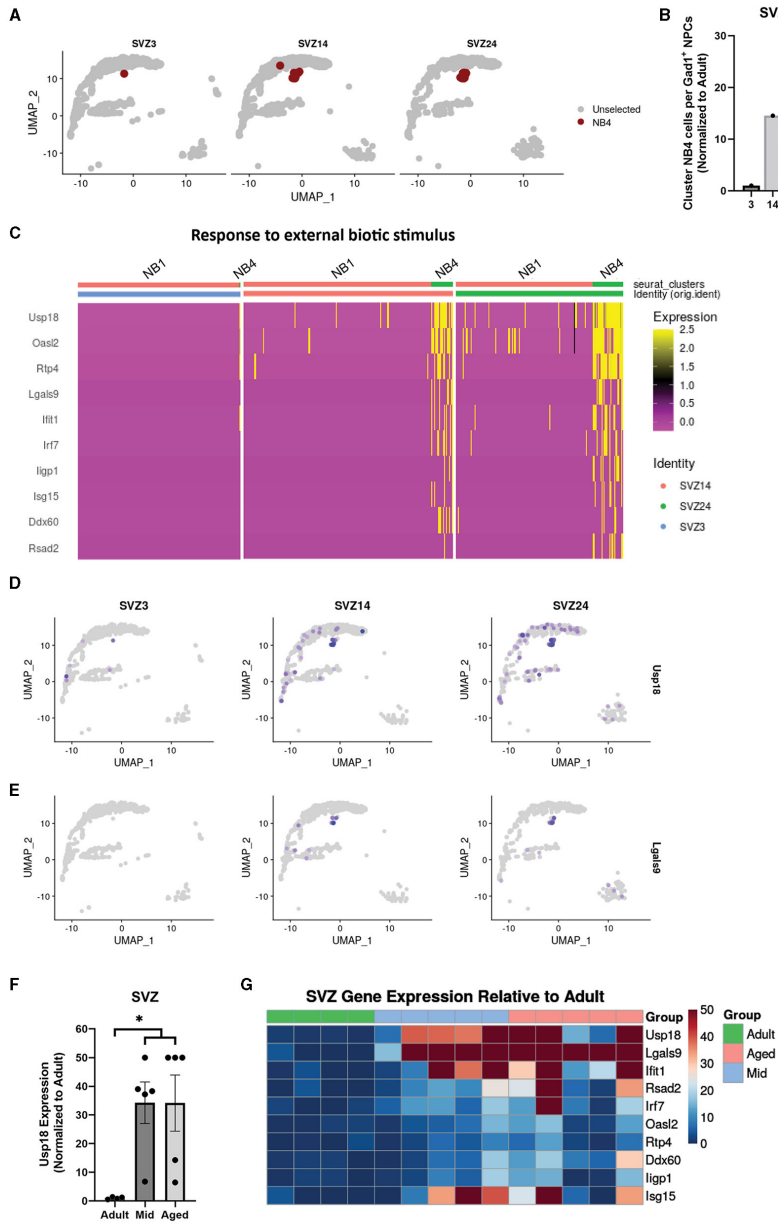

FIGURE 4
A distinct population of neuroblasts in the aged SVZ acquires an expression profile related to immune responses. Uniform manifold approximation and projection (UMAP) highlighting cluster NB4 in different age groups of the SVZ **(A).** Quantification of cells in cluster NB4 and non-cycling cell populations expressing Gad1 (clusters NB1, NB2, and NB3) in the SVZ, represented as a ratio **(B)**. Heatmap showing differentially expressed genes between cluster NB4 and a combined cluster of NB1, NB2, and NB3 for genes with the common gene ontology term "response to external biotic stimuli," all related to immune response **(C).** Uniform manifold approximation and projection (UMAP) showing expression of genes defining cluster NB4 in the SVZ, Usp18 **(D)** and Lgals9 **(E).** Gene expression of Usp18 normalized to adult in bulk sequencing samples of the SVZ, *p.adj of < 0.05, with DESeq2, of adult samples compared to a combined group of middle-aged and aged samples **(F).** Heatmap showing gene expression of top defining genes of cluster NB4 from different bulk samples relative to mean adult in the SVZ **(G).**

neurons (Figure 3A). This previously described subpopulation of neural progenitors (Sequerra et al., 2013) has not been well studied in the context of aging.

The number of OPCs has shown to remain fairly constant during aging (Wang et al., 2020), while other large populations in our dataset varied. We, therefore, used OPCs as a reference to analyze how the SVZ and DG IP populations changed in size with aging. Interestingly our single cell data suggested that the population of Eomes (Tbr2) positive IPs, in both neurogenic regions, decreased more rapidly with age compared to IPs positive for Gad1 (Figures 3B–E).

To test this statistically, we sorted GFP-positive cells from adult, middle-aged, and aged SVZ of Dcx-GFP mice, performed bulk mRNA sequencing, and analyzed markers specific for the different IP populations. Our bulk data, indeed, showed that the expression of Eomes (Tbr2), Tbr1, and Slc17a6 (Vglut2) decreased more rapidly with age in the SVZ and DG compared to Gad1 and St18 in the SVZ (Figures 3F–H).

In summary, our RNA expression data suggest that the number of IPs set to become excitatory neurons, regardless of the neurogenic region, decreases faster during aging than that of IPs set to become inhibitory interneurons.

3.3 A distinct population of neuroblasts in the aged SVZ acquires an expression profile related to immune responses

Having shown that most aged neural progenitors retain their expression profiles with minor differences, while the composition of neuronal progenitor subtypes changes, we next investigated whether any IPs acquire new cellular identities during aging. Concomitant with decreased neurogenesis, inflammation in the brain increases (Ferrucci and Fabbri, 2018). However, it is unknown whether aged IPs change their gene expression related to immune responses and whether such changes are global or selective.

Since we did not find any major age-related gene expression differences in clusters where population size was unchanged (Figures 2A, B), we decided to analyze cluster NB4 that clearly increased in size in the SVZ with age (Figures 4A, B) and was not detectable at any age in either OB or DG. Interestingly, compared to Gad1+ IPs (NB1, NB2, and NB3), NB4 exhibited a gene expression pattern similar to neuroblasts but with several upregulated genes involved in innate immune responses and responses to external biotic stimuli (Figure 4C). By exploring the differentially expressed genes (Supplementary Table 1), we found Usp18 (Ubiquitin Specific Peptidase 18) to be highly enriched in cluster NB4 and also detected its presence to a greater extent in other cells from aged samples (Figure 4D). Usp18 is induced by viral infection and type I interferons and inhibits apoptosis (Malakhova et al., 2006; Diao et al., 2020). In addition, we found Lgals9 (Galectin-9), a suppressor of T-cells (Yang et al., 2021), to be specific to cluster NB4 (Figure 4E). Therefore, we analyzed Usp18 and Lgals9, along with other top genes defining cluster NB4 (Supplementary Table 1), in our SVZ bulk data and found an increased expression of most of these immune response genes in the SVZ from the middle-aged and aged samples compared to those in adult samples (Figures 4F, G).

In summary, a large portion of IPs in the aged neurogenic niches exhibit only minor changes in gene expression that can be linked to immune responses, while a small selection of IPs in the SVZ not detected in the OB acquire a distinct expression profile related to immune responses.

4 Discussion

In this study, we generated a unique dataset and performed the first detailed transcriptional analysis of highly enriched IPs from the primary murine neurogenic niches during aging. The single-cell sequencing data are supported by bulk sequencing data to include biological variance; however, they did not yield individual animal resolution due to the challenges in obtaining a sufficient number of purified IPs from single animals.

We demonstrated that most aged IPs undergo only minor transcriptional changes, some of which are non-linear, cell-type specific, or shared, but none are universal. In addition, we found that neuroblasts set to become excitatory neurons decreased faster than IPs set to become inhibitory neurons in both neurogenic niches. Furthermore, some progenitors in the aged SVZ, not present in the OB or adult animals, showed a strong immune reactive expression profile.

Aging is a heterogeneous process that varies between individuals (Somel et al., 2006; Lowsky et al., 2014) and has been reported to drive transcriptional paths differently between cell types (Ximerakis et al., 2019). We found that aging affect gene expression differently not only between cell types but also between transient cell states of neuroblasts, albeit to a lesser extent. This indicates that the currently active transcriptional program is responsible for determining which genes are vulnerable to aging-induced expression changes. Epigenetic regulation could potentially explain these findings as DNA methylation, histone modification, chromatin remodeling, non-coding RNA regulation, and RNA modification all participate in the aging process (Wang et al., 2022) and have the potential to project transcriptional changes differently, based on the currently active program.

As anticipated based on the previous literature, we observed differentiation trajectories from NSCs to excitatory neurons in the DG, to both inhibitory and excitatory neurons in the SVZ, and only to inhibitory neurons in the OB. The NSC population and the subsequent differentiation to inhibitory neurons in the OB represent an intriguing area of study because it has received less attention. This process might be differentially affected by aging as compared to neurogenesis occurring in the SVZ. However, our experiments were designed to analyze neuroblasts, not NSCs and neurons; therefore, our data do not have enough power to make any solid conclusions regarding OB NSCs, which should be addressed in separate studies.

Interestingly, we found a few genes, mainly ribosomal, that exhibited non-monotonic changes during aging, with maximum expression in the middle-aged group of several cell types in the SVZ. The increased expression of ribosomal genes in the middle-aged group reverted to adult levels in aged animals. Alterations in the ribosomes in middle age can potentially lead to a translation imbalance, contributing to the development of aging phenotypes. Such patterns can also be an attempt to rescue or compensate for aging phenotypes before they become detrimental.

A previous study of the SVZ used bulk RNA sequencing to detect non-monotonic changes during aging. This study found that the expression of most genes exhibited such patterns, with both the minimum and maximum levels of gene expression occurring at 18 months (Apostolopoulou et al., 2017). However, bulk sequencing reflects cell type abundance and expression of the same gene in different cell types, which can be regulated in different directions during aging (Ximerakis et al., 2019). Our data confirm that non-monotonic changes can occur but suggest that they are less common and not often universal.

In most aged SVZ neuroblasts, we detected only a few transcriptional changes, some of which were involved in axonogenesis. This finding suggests that most aged neuroblasts still have the potential to generate functional neurons, possibly with altered axon development. Supporting this observation, others have reported that new DG neurons can integrate into the old brain, albeit with compromised dendrite development (Trinchero et al., 2017). Among the upregulated genes in aged neuroblasts, Foxg1 suppresses differentiation (Martynoga et al., 2005) and decreases with maturation (Miyoshi and Fishell, 2012), while Tubb3, which is downregulated in aged neuroblasts, promotes differentiation (Cao et al., 2017) and increases with maturation until it peaks (Hausrat et al., 2021). This observation suggests slower maturation of aged SVZ neuroblasts compared to their adult counterparts. Foxg1 has also been reported to assist in directing toward certain neuron subtypes through Nr4a2 (Ba et al., 2023) and to shift toward a GABAergic inhibitory neuron identity (Mariani et al., 2015). This suggests that neuroblasts in the aged SVZ may produce different composition of neuron subtypes compared to adult neuroblasts.

NSCs in the adult DG and SVZ predominantly produce different types of neurons (Jurkowski et al., 2020). However, it has been suggested that a minor population of spatially defined NSCs in the SVZ give rise to excitatory neurons (Sequerra, 2014) similar to those generated in the DG. Our data showed that IPs of excitatory neurons decrease more drastically in both neurogenic regions, as previously indicated in the DG (Galvan and Jin, 2007). This finding suggests that the differences in aging-related neurogenic decline between the SVZ and that DG are not due to major regional tissue differences but are rather due to cell-type specific and dependent on cell-intrinsic transcriptional programs. Future studies should assess whether SVZ NCSs that give rise to excitatory progeny are more similar to DG NSCs, whether their activity decreases or changes as they age, whether they start generating progenitors with inhibitory potential, or whether neuroblasts with excitatory potential are pushed toward an inhibitory identity in the aged SVZ. As our observations are based purely on single-cell transcriptomic changes, which were validated using bulk data, it is important to develop robust methods to detect these and other discrete populations at the protein level *in vivo*.

While most IPs only acquired minor changes with age, we found a population of neuroblasts that displayed a strong immune-responsive gene expression profile appearing in the middle-aged and aged SVZ. This population is defined by Lgals9 expression, which is increased in all middle-aged and aged biological SVZ samples. This population was not detected in the OB, which strongly suggests that these neuroblasts, for unknown reasons, are inhibited from reaching their destination and mature into new neurons. This process could potentially be a mechanism to select for healthy neuroblasts, yet it could also be an adverse reaction due to an altered inflammatory milieu in the neurogenic niche.

While most IPs only underwent minor changes with age, we found a population of neuroblasts in the aged SVZ displaying a strong immune-responsive gene expression profile that appeared in middle-aged and aged samples. This population, defined by Lgals9 expression, was increased in all middle-aged and aged SVZ samples and was not detected in the OB, suggesting that these neuroblasts are inhibited from reaching their destination and maturing into new neurons. This inhibition could potentially be a mechanism to select for healthy neuroblasts but could also be an adverse reaction to an altered inflammatory milieu in the neurogenic niche.

It has been previously reported that an inflammatory environment modulates NSC stemness through TNF-α (Belenguer et al., 2021). TNF-α expression increases in the aged neurogenic niche, contributing to reduced neurogenesis (Fonseca et al., 2016). We did not observe any signs of altered TNF-α signaling in neuroblasts; instead, our data indicated altered interferon signaling, which, like TNF-α, is suggested to preserve stemness and restrict differentiation (Sato et al., 2020; Ibañez et al., 2023). Although limited data exist on neuroblasts, the observed interferon-related immune response may have a similar effect, contributing to fewer neuroblasts differentiating into mature neurons. In addition, we cannot exclude the possibility that the observed effects on neuroblasts are mediated through TNF-α signaling on parental NSCs, which is carried over to differentiating neuroblasts.

The immune response in neuroblasts could be triggered by increased infiltration of immune cells from the periphery. Supporting this idea, T-cell infiltration into the SVZ increases with age (Dulken et al., 2019; Zhang et al., 2022b), and Lgals9 acts as a suppressor of T-cells (Yang et al., 2021). This likely contributes to the reduced number of new neurons in the aged OB. It would be interesting to experimentally ablate infiltrating immune cells, specifically T-cells, to analyze if the reactive neuroblast population still appears in the aged SVZ. Furthermore, understanding whether this population is long-lived and has functional implications on neighboring cells is crucial. Previous studies have shown that removing senescent cells can lead to rejuvenation (Zhang et al., 2022a), making it appealing to further study and attempt to deplete this immune-reactive neuroblast population.

Data availability statement

The data presented in the study are deposited in the Gene Expression Omnibus (GEO) repository, accession number GSE261459, which can be found at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE261459.

Ethics statement

The animal study was approved by Malmö/Lund Ethics Committee on Animal Testing at the Lund District Court. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

JF: Writing – original draft, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Project administration, Resources, Validation, Visualization. SL: Conceptualization, Data curation, Formal analysis, Writing – original draft, Writing – review & editing. MS: Investigation, Writing – original draft, Writing – review & editing. SS: Data curation, Resources, Writing – original draft, Writing – review & editing. HA: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fnins.2024.1400963/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Experimental design for single-cell RNA sequencing, showing Dcx+ IPs in the DG, SVZ, and OB neurogenic regions of adult, middle-aged, and aged mice and sorting of Dcx+ cells before library preparation and sequencing **(a)**. Violine plots showing cells retained after quality control, with (from left) more than 75 and <4,000 detected genes, <3,500 molecules, <1.5% mitochondrial genes, and <15% ribosomal genes **(b)**. Highly variable genes used for clustering **(c)**. Trajectories, from immature to mature states, of cells from the neuronal linage in the SVZ **(d)**, OB **(e)**, and DG **(f)**.

SUPPLEMENTARY FIGURE 2
Barplot showing total reads per strand per sample **(a)**, percentage of read duplication **(b)**, percentage of GC content **(c)**, and the total number of paired reads, number of reads aligned one time, and more than one time **(d)**.

SUPPLEMENTARY FIGURE 3
Boxplot showing Cook's test for marker genes for excitatory neurons **(a)**, and genes connected to inflammation **(b)**, with larger numbers indicating outliers.

SUPPLEMENTARY TABLE 1
Grubbs' test for genes connected to inflammatory responses and markers for excitatory neurons. The sample (row title) shows the sample farthest from the mean and considered an outlier when p-value is <0.05.

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