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**INVESTIGATING MICROGLIA-VASCULAR INTERACTIONS IN THE
DEVELOPING AND ADULT CENTRAL NERVOUS SYSTEM**

A Dissertation Presented

by

Erica Mondo

Submitted to the Faculty of the

University of Massachusetts Graduate School of Biomedical Sciences, Worcester

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 26th, 2020

NEUROBIOLOGY

**INVESTIGATING MICROGLIA-VASCULAR INTERACTIONS IN THE
DEVELOPING AND ADULT CENTRAL NERVOUS SYSTEM**

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Erica Mondo

This work was undertaken in the Graduate School of Biomedical Sciences

Program of Neuroscience

Under the mentorship of

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ABSTRACT

Microglia, the resident macrophages of the central nervous system (CNS), are dynamic cells, constantly extending and retracting their processes as they contact and functionally regulate neurons and other glial cells. There is far less known about how microglia interact with the CNS vasculature, particularly under healthy steady-state conditions. Here, I provide the first extensive characterization of juxtavascular microglia in the healthy, postnatal brain and identify a molecular mechanism regulating the timing of these interactions during development. Using the mouse cerebral cortex, I show that microglia are intimately associated with the vasculature in the CNS, directly contacting the basal lamina in vascular sites that are devoid of astrocyte endfeet. I demonstrate a high percentage of microglia are associated with the vasculature during the first week of postnatal development, which is concomitant with a peak in microglial colonization of the cortex and recruitment to synapses. I find that as microglia colonize the cortex, juxtavascular microglia are highly motile along vessels and become largely stationary as the brain matures. 2-photon live imaging in adult mice reveals that these vascular-associated microglia in the mature brain are stable and stationary for several weeks. Further, a decrease in microglia motility along the vasculature is tightly correlated with the expansion of astrocyte endfeet along the vasculature. Finally, I provide evidence that the timing of these microglia-vascular interactions during development is regulated by the microglial fractalkine receptor (CX3CR1). Together, these data support a model by which

microglia use the vasculature as a scaffold to migrate and colonize the developing brain and the timing of these associations is modulated by CX3CR1. This migration along the vasculature becomes restricted as astrocyte vascular endfoot territory expands and, upon maturation, vascular-associated microglia become largely stationary.

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List of symbols, abbreviations, or nomenclature

Aβ	β -amyloid
ACSF	Artificial cerebral spinal fluid
AIS	Axon initial segment
AD	Alzheimer's disease
AMPA	α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid
AQP4	Aquaporin 4
ASDs	Autism spectrum disorders
ATP	Adenosine triphosphate
BBB	Blood brain barrier
BDNF	Brain-derived neurotrophic factor
BSA	Bovine serum albumin
C1q	Complement component 1q
CBF	Cerebral blood flow
CNS	Central nervous system
COX	Cyclooxygenase
CSF1R	Cell survival factor 1 receptor
CX3CL1	Fractalkine
CX3CR1	Fractalkine receptor
CXCL	Chemokine (C-X-C motif) ligand
CXCR	Chemokine (C-X-C motif) receptor
E	Embryonic day
EAE	Experimental autoimmune encephalomyelitis
ECM	Extracellular matrix
EGFP	Enhanced green fluorescent protein
EM	Electron microscopy
ExM	Expansion microscopy
FGF2	Fibroblast growth factor 2
FOV	Field of view
GABA	Gamma-aminobutyric acid
GFP	Green fluorescent protein
GPCR	G-protein-coupled receptor
GPR124	G protein-coupled receptor 124
GW	Gestational week
HBSS	Hank's balanced salt solution
HUVEC	Human umbilical vein endothelial cell
IBA-1	Ionized calcium-binding adaptor molecule 1
ICAM	Intracellular adhesion molecule
IFN	Interferon
IGF-1	Insulin growth factor 1
IL	Interleukin
iNOS	Inducible nitric oxide synthase
IRF8	Interferon regulatory factor 8

LIF	Leukemia inhibitory factor
LPS	Lipopolysaccharide
LYVE1	Lymphatic vessel endothelial receptor 1
MCP-1	Chemoattractant protein 1
MERFISH	Multiplexed error-robust fluorescence in situ hybridization
MIA	Maternal immune activation
MMPs	Metalloproteinases
MS	Multiple sclerosis
NFκB	Nuclear factor-kappa B
NMDA	N-methyl-d-aspartic acid
NO	Nitric oxide
NPCD	Normal programmed cell death
NPCs	Neural progenitor cells
NRP1	Neuropilin 1
NVU	Neurovascular unit
OPCs	Oligodendrocyte precursor cells
P2RY12	Purinergic receptor P2Y12
P	Postnatal day
P75NTR	Neurotrophin receptor P75
PB	Phosphate buffer
PBS	Phosphate buffered saline
PDGFRβ	Platelet derived growth factor receptor β
PECAM	Platelet endothelial cell adhesion molecule
PFA	Paraformaldehyde
PGE	Prostaglandin E2
PI-3	Phosphatidylinositol-3
PNVP	Perineural vascular plexus
RNA-seq	RNA-sequencing
ROI	Region of interest
ROS	Reactive oxygen species
rOTO	Reduced osmium-thiocarbohydrazide-sodium
SDF-1	Stromal cell derived factor 1
SEM	Scanning electron microscopy
SIM	Structured illumination microscopy
SIRT1	Sirtuin 1
SMA	Smooth muscle actin
SPP1	Osteopontin
SVZ	Subventricular zone
TGF-β	Transforming growth factor β
TNF	Tumor necrosis factor
TNF-α	Tumor necrosis factor alpha
Trkβ	Tyrosine receptor kinase β
TX-100	Triton-X100
UFP	Ultrafine particles

VAST	Volume and segmentation tool
VEGF	Vascular endothelial growth factor
VEGFR-1	Vascular endothelial growth factor receptor 1
VGlut2	Vesicular glutamate transporter 2
WT	Wild type

List of multimedia objects

Multimedia objects in Chapter II

Movie 1: 3D rendering of juxtavascular microglia in the early postnatal frontal cortex. 3D reconstruction and surface rendering of juxtavascular microglia (green, EGFP) associated with blood vessels (magenta, anti-PECAM) in the P5 frontal cortex. Yellow denotes association area between microglia and blood vessels.

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Preface

The work presented in **Chapter II** is previously published.

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The work presented in **Chapter III** is unpublished.

Erica Mondo, Shannon C Becker, Sergio A. Lira, and Dorothy P. Schafer.

"The fractalkine receptor regulates the timing of microglia-vasculature interactions independent of fractalkine."

Contributions of the authors are addressed at the beginning of each chapter.

CHAPTER 1: GENERAL INTRODUCTION

Microglia in the Healthy Central Nervous System

Microglia, the resident immune cells and phagocytes of the central nervous system (CNS), were first described in the mammalian CNS by Pio del Rio Hortega nearly a century ago (Rio-Hortega 1932). Since their discovery, the vast majority of studies have focused on their role in the diseased and injured CNS, in which they perform a broad range of functions such as shielding injury sites, phagocytosing cellular material, and releasing inflammatory signals to initiate and/or propagate the immune response (Wyss-coray and Mucke 2002; Napoli and Neumann 2009; Ransohoff and Perry 2009; Ransohoff and Cardona 2010; Kettenmann et al. 2011; Sierra et al. 2013; Schafer, Lehrman, and Stevens 2013). However, in recent years there has been a growing appreciation for the importance of microglia in the healthy CNS. Seminal 2-photon *in vivo* live imaging studies have demonstrated that “resting” microglia in the healthy, adult cerebral cortex are highly active, continuously extending and retracting their processes (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005). These processes are frequently found in direct contact with neurons, synapses, and other glial cell, raising questions about what other CNS cell types microglia could be contacting in the healthy developing and adult brain (Schafer et al. 2012; Tremblay, Lowery, and Majewska 2010; Cserép et al. 2020). Microglia are known to contact the brain vasculature, however, the vast majority of these studies have been in the context of disease where parenchymal microglia rapidly

associate with the brain vasculature following breakdown of the blood-brain barrier (BBB) (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018). Far less is known about how microglia interact with the vasculature in the healthy brain. In this thesis, I investigate juxtavascular microglia through postnatal development and adulthood, explore a role for the vasculature in microglial colonization, and identify a molecular mechanism regulating the timing of these interactions during development. This work advances our understanding of juxtavascular microglia and lays the fundamental groundwork to investigate the function of these cells in the healthy and diseased brain.

The origins of microglia

Unlike the vast majority of cells found in the CNS, microglia are of mesodermal origin. In rodents, a subset of CD45⁺ c-kit⁺ erythromyeloid precursors use the blood circulation to travel from the embryonic yolk sac to the mesenchyme surrounding the neural tube beginning at embryonic day (E) 8 (Alliot, Godin, and Pessac 1999; Kierdorf et al. 2013). Upon colonization of the mesenchyme, these microglial precursors downregulate c-kit, upregulate the fractalkine receptor (CX3CR1), and invade the neuroepithelium at E9.5 by crossing the pial surface and lateral ventricles (Kierdorf et al. 2013; Ginhoux et al. 2010; Navascués et al. 2000; Swinnen et al. 2013). The initial formation and survival of microglial precursors is dependent on cell survival factor 1 receptor (CSF1-R) signaling, the transcription factors PU.1 and interferon regulatory factor

8 (IRF8) (Kierdorf et al. 2013; Ginhoux et al. 2010; Erblich et al. 2011). Once established in the CNS, microglia then migrate and proliferate through the brain parenchyma in a rostral-to-caudal gradient to colonize the embryonic brain (Sorokin et al. 1992; Navascués et al. 2000; Swinnen et al. 2013; Alliot, Godin, and Pessac 1999; Perry, Hume, and Gordon 1985; Ashwell 1991). In humans, microglia enter and colonize the embryonic brain during early gestational weeks (GW) in a similar manner. Human amoeboid microglia precursors enter into the brain rudiment from the leptomeninges, the ventricular lumen, and the choroid plexus at GW4.5-5.5, then colonize in a radial and tangential manner towards the immature white matter, subplate layer, and cortical plate (Verney et al. 2010; Monier et al. 2007; Andjelkovic et al. 1998).

In rodents, signaling mechanisms have been identified to regulate the initial infiltration of microglial precursors into the brain parenchyma, such as matrix metalloproteinases (MMPs), stromal cell derived factor 1 (SDF-1), and chemokine (C-X-C motif) ligand (CXCL) 12/ chemokine (C-X-C motif) receptor (CXCR) 4 signaling (Ginhoux et al. 2010; Arno et al. 2014; Ueno and Yamashita 2014). However, far less is known about the mechanisms regulating microglial localization to the appropriate brain regions once they reach the parenchyma, particularly during postnatal development. Remarkably, during both rodent and human embryonic development, microglia are found localized to the brain vasculature, but these interactions have not been explored in the rodent postnatal brain (Verney et al. 2010; Monier et al. 2007; Fantin et al. 2010;

Smolders et al. 2017; Checchin et al. 2006). In Chapter II, I demonstrate that microglia are highly associated with the vasculature and migrate on blood vessels, concomitant with colonization of the postnatal brain. This work suggests a mechanism by which microglia use the vasculature to colonize the developing brain parenchyma, providing a foundation to investigate the precise molecular pathways regulating vascular-dependent microglial localization to the appropriate brain regions. Understanding how microglia colonize the brain will provide insight into several neurodevelopmental disorders, such as autism spectrum disorders (ASDs), where changes in microglial densities have been described (Edmonson, Ziats, and Rennert 2016; Morgan et al. 2010).

Microglial interactions with synapses, neurons, and glia

Once established in the brain, microglia continually survey their local environment by extending and retracting their processes, making transient contacts with synapses, dendritic spines, neuronal cell bodies, and astrocytes (Wake et al. 2009; Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005; Tremblay, Lowery, and Majewska 2010). Early *in vivo* 2-photon live imaging has revealed that microglial processes briefly contact synaptic elements in layers II-III of the somatosensory and visual cortices at a rate of ~1 structure per hour (Feng et al. 2000; Hirasawa et al. 2005; Wake et al. 2009). A more recent study used a combination of high-resolution 3D serial electron microscopy, with 2-photon transcranial imaging to attain a better spatial

resolution of microglial contacts with synapses and found that microglia in layer II of the visual cortex contacted dendritic spines, synaptic terminals, and synaptic clefts (Tremblay, Lowery, and Majewska 2010). Importantly, several studies have demonstrated that after microglial contact, dendritic spines often change in size or will appear in sites of microglia-synapse contact, suggesting that this contact may be key regulator of structural spine plasticity (Tremblay, Lowery, and Majewska 2010; Miyamoto et al. 2016; Weinhard et al. 2018). In addition to synapses and dendritic spines, microglia are found to make direct contacts with >90% of neuronal cell bodies in both the rodent and human brain (Cserép et al. 2020). Neuronal cell body-microglia junctions sites have a specialized nanoarchitecture optimized for purinergic signaling (Cserép et al. 2020). As purinergic signaling is key for cell-to-cell communication between neurons and microglia, this suggests that microglia contacts at these junctions could monitor and protect neuronal functions (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005; Cserép et al. 2020). Finally, in the cortex, but not other brain regions, a subset of microglia extend a single process that specifically associates and overlaps with the neuronal axon initial segment (AIS) (Baalman et al. 2015). Microglia-AIS interactions occur early in development, persist throughout adulthood, and are thought to play roles in healthy brain functions as these interactions are lost after brain injury (Baalman et al. 2015).

In addition to neurons, microglia are found in direct contact with other glial cells, including astrocytes. Confocal microscopy studies reveal that microglia

make direct soma-soma contacts, as well as process-process interactions with astrocytes in the hippocampus (Jinno et al. 2007). Further ultrastructural analysis of these contact sites in the visual cortex demonstrates that microglial processes directly interact with astrocyte processes at synapses and changes in synaptic activity leads to changes to the percent contact between microglia and astrocytes (Tremblay, Lowery, and Majewska 2010). Given that both astrocytes and microglia play complementary roles in support of synapse formation and remodeling, by communicating directly through cytokines and other molecules, these contact sites at synapses are an ideal location for communication between microglia and astrocytes (Vainchtein and Molofsky 2020).

It is well established that microglial processes are highly active and motile in the healthy brain, contacting several different CNS cell types and structures as they survey the parenchyma. However microglial contacts with the vasculature have yet to be explored in this context. In Chapter II, I determine that microglia somas contact the vasculature throughout the developing postnatal and adult brain. As microglial contacts with other cell types often lead to functional changes or are sites of communication, this data suggest that microglia may regulate, and directly communication, with the vasculature in the healthy brain.

Microglial functions in the developing CNS

The developing CNS represents a dynamic period in the lifetime of an organism that requires coordination and communication amongst several

different cell types. The direct physical contacts that microglia make with neurons, synapses, and other glial cell places microglia in the unique position to play critical roles in CNS development (Schafer et al. 2012; Tremblay, Lowery, and Majewska 2010; Cserép et al. 2020). For example, microglia regulate neuronal cell numbers in the developing brain by releasing factors that promote the survival, proliferation, and maturation of neural progenitor cells (NPCs), as well as by engulfing dead or dying cells that have undergone normal programmed cell death (NPCD) (Ferrer et al. 1990; Bessis et al. 2007). Specifically, mice deficient in the fractalkine receptor (CX3CR1), a chemokine receptor enriched in microglia, have significant increases in the numbers of apoptotic neurons in layer V of the postnatal cortex, which is attributed to reductions in insulin-growth factor 1 (IGF-1) signaling, a potent trophic factor for NPC survival (Ueno et al. 2013; Wolf et al. 2013; Mizutani et al. 2012; Nishiyori et al. 1998). Moreover, in cultured rat cerebellar slices, microglia engulf dead or dying cells and pharmacological depletion of microglia results in reduced Purkinje neuron NPCD, suggesting microglia can actively initiate the cell death program (Martin-Teva et al. 2004).

In addition to regulating neuronal cell numbers, microglial are critical regulators of synapse development and maturation. In the developing CNS, neurons initially form a crude synaptic wiring diagram. Through a neuronal activity-dependent manner, these synaptic connections are refined, whereby less active connections are eliminated and more active connections are maintained and strengthened (Katz and Shatz 1996; Hua and Smith 2004). Live imaging

studies have revealed that microglia can sense and respond to changes in neuronal activity, through neuronal activity-dependent release of adenosine triphosphate (ATP) (Dissing-Olesen et al. 2014; Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005). Further, modulating neuronal activity by rearing mice in the dark and re-exposing them to light induces changes in the frequency and duration of microglial contact with synapses in the visual cortex, suggesting that microglia regulate synapse development through activity-dependent mechanisms (Tremblay, Lowery, and Majewska 2010; Wake et al. 2009). Indeed, studies in the developing mouse retinogeniculate system have shown that microglia eliminate synaptic connections by engulfing a subset of immature, less active presynaptic inputs through complement mediated phagocytosis (Schafer et al. 2012). Further, microglia mediated synaptic remodeling occurs in response to sensory loss and dampened neuronal activity in the developing rodent barrel cortex (Gunner et al. 2019). In addition to regulating synapse development, microglia play a role in the maturation of synaptic connections. A transient reduction in microglia numbers in the hippocampus or barrel cortex, due to a genetic deletion of CX3CR1, results in delayed maturation of structural and functional synapses (Paolicelli et al. 2011; Hoshiko et al. 2012). These effects are attenuated in juvenile mice when microglial density in CX3CR1 deficient mice reaches wild-type levels. However, in adulthood, these mice display phenotypes associated with ASDs, including decreased functional brain connectivity, deficits in social interactions, and

increased repetitive behaviors (Zhan et al. 2014), suggesting a critical role for microglia in the development of mature functional neuronal circuits.

Microglia also play a role in regulating non-neuronal cell development. *In vitro* evidence suggests that microglia-conditioned media promotes the survival and differentiation of cultured oligodendrocyte precursor cells (OPCs) into mature oligodendrocytes through secretion of IGF-1, nuclear factor-kappa B (NF κ B), interleukin (IL)- β and IL-6 (Shigemoto-Mogami et al. 2014; Lu et al. 2013; O'Kusky and Ye 2012; Nicholas, Wing, and Compston 2001). Microglia have also been suggested to promote myelination by providing iron, a necessary co-factor for myelination, to oligodendrocytes (Clemente et al. 2013; X. Zhang et al. 2006; Cheepsunthorn, Palmer, and Connor 1998). Finally, it has been shown that microglia-conditioned media increases the differentiation of NPCs into astrocytes through IL-6 and leukemia inhibitory factor (LIF) (Nakanishi et al. 2007). While it is unknown if these mechanisms apply *in vivo*, microglia may have the potential to regulate the survival and differentiation of other glial cells.

Microglial localization and direct contact with neurons, synapses, and other glial cells places microglia in an ideal position to regulate their development through engulfment and release of factors to promote cell survival and differentiation. In this thesis, I demonstrate that a high percentage of microglia are juxtavascular during CNS development. This raises the question, what role do juxtavascular microglia play at the vasculature? Evidence in the embryonic brain suggests that microglia play a role in promoting vascular branching (Fantin

et al. 2010). Contrary to this, in Chapters II and III I demonstrate that delays in microglial localization to the vasculature do not result in changes to vascular density. Moreover, previous studies find that microglial depletion in the postnatal brain leads to no change in the vasculature (Parkhurst et al. 2013; Elmore et al. 2014). Thus, a deeper understanding of juxtavascular microglia in the developing and healthy postnatal brain is required and will provide the groundwork to investigate the function of juxtavascular microglia in the healthy brain, which I explore in Chapter IV.

The Brain Vasculature

The brain vasculature consists of a multicellular unit, the neurovascular unit (NVU), which connects the brain parenchyma to the cerebral vasculature. Large arteries that penetrate from the subarachnoid space consist of endothelial cells, basal lamina, smooth muscle cells, perivascular macrophages, and astrocyte endfeet (McConnell et al. 2017). As vessels dive deeper into the brain and become capillaries, smooth muscle cells are lost and pericyte coverage takes their place (Fig 1.1 A-B) (McConnell et al. 2017). Interactions between these NVU cell types is important for a variety of physiological processes such as angiogenesis, vessel maintenance and permeability, metabolic support, and regulation of blood flow (L. S. Brown et al. 2019; McConnell et al. 2017). While microglia are often described as a cellular compartment of the NVU, the precise spatial and functional relationship between microglia and the NVU remains an

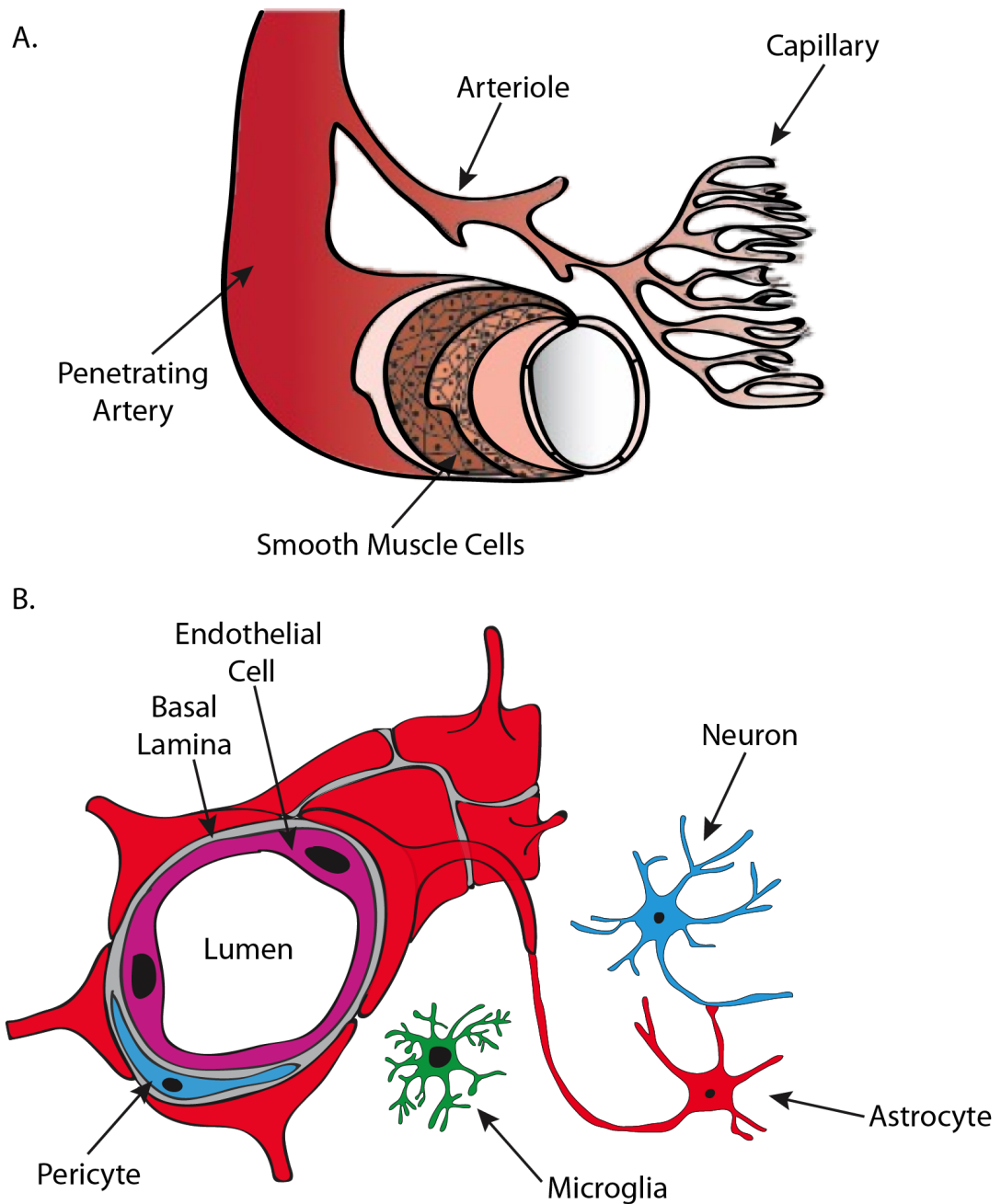


Figure 1.1: The brain Vasculature. **A.** Large penetrating arteries enter the brain parenchyma from the subarachnoid space, which then branch into smaller arterioles and then form a dense capillary network. Penetrating arteries are distinguished from other vessels by the presence of smooth muscle cells. **B.** The cells that form the vasculature, together known as the neurovascular unit, consist of endothelial cells, pericytes, basal lamina, astrocyte, neurons, and microglia.

open question. In this thesis, I determine precisely where within the NVU microglia associate with vessels and explore a role for cells of the NVU in regulating microglial migration during brain colonization, advancing our understanding of the cellular components of the NVU and the role it plays in CNS development.

NVU development

The development of the NVU begins via the process of vasculogenesis, whereby mesoderm-derived angioblasts invade the head region and merge to form the perineural vascular plexus (PNVP), a primitive vascular network that covers the entire surface of the neural tube at E7.5-8.5 (Hogan et al. 2004). Beginning at E9.5, nascent endothelial cells originating from the PNVP invade the neural tube, elongate toward the ventricular zone, form a series of lateral branches, and anastomose with adjacent vascular sprouts to produce a plexus of capillaries (Engelhardt 2003; Engelhardt and Liebner 2014; Saili et al. 2017). Pericytes, a mural cell that sits on the abluminal surface of the endothelial cell embedded within the basal lamina, associate with endothelial cells as the nascent vessels generate as early as E10 (Armulik et al. 2010; Bauer et al. 1993; Yamanishi et al. 2012; Daneman et al. 2010; Zlokovic 2008). Interactions between pericytes and endothelial cells are critical to form the BBB (Daneman et al. 2010; Armulik, Genové, and Betsholtz 2011; L. S. Brown et al. 2019). Astrocytes are also a key component of the NVU, however they do not appear in

the cortex until early postnatal development, when they extend their processes to form endfeet on developing vasculature (Daneman et al. 2010). These astrocyte endfeet ultimately surround and ensheath the majority of the vasculature by adulthood where they play roles in a variety of functions such as maintaining the BBB, providing metabolic support to neurons, and regulating blood flow (Abbott, Rönnbäck, and Hansson 2006; Kimelberg and Nedergaard 2010; Macvicar and Newman 2015). Once developed, the cells that make up the NVU create an important cellular barrier that tightly controls the microenvironment and the exchange of nutrients between the cerebral blood flow and the CNS.

Previous work demonstrates that microglia colonize the brain precisely when nascent endothelial cells begin infiltrating the neuroepithelium, E9.5, and microglia are found associated with vessels during embryonic development, which is discussed in greater detail below (Ginhoux et al. 2010; Engelhardt 2003; Engelhardt and Liebner 2014; Saili et al. 2017; Fantin et al. 2010; Smolders et al. 2017). Whether microglia are part of the developing NVU in the postnatal brain, and what cells types they associate with, remain open questions. In Chapter II, I demonstrate that microglia are juxtavascular in the healthy postnatal and adult brain and simultaneously contact the basal lamina and astrocyte endfeet across development. Additionally, I propose a mechanism by which astrocyte endfeet expansion may exclude juxtavascular microglia from contacting blood vessels. This work addresses a critical need to understand the cellular components of the NVU, as the NVU plays an essential role in brain homeostasis and becomes

dysfunctional in several neurodegenerative disorders (Zhao et al. 2018; Stankovic, Teodorczyk, and Ploen 2016; McConnell et al. 2017).

The brain vasculature as a scaffold for cell migration

The development of the brain vasculature and the NVU is not only important for proper communication between the brain parenchyma and the cerebral blood flow, it also serves as a scaffold for migration of several different cell types in the healthy brain. In the embryonic brain, gamma-aminobutyric acid (GABA) inhibitory interneurons are associated with blood vessels as they migrate towards the cortex. During adult neurogenesis, subventricular zone (SVZ)-derived neuroblasts migrate tangentially towards the olfactory bulb along blood vessels (Bovetti et al. 2007; Snayyan et al. 2009; Whitman et al. 2009). Additionally, another glial cell type, OPCs, migrate on blood vessels during embryonic development and utilize vessels to reach the appropriate brain location (Tsai et al. 2016). In the embryonic brain, microglia have been described to be in close contact with the vasculature and even display saltatory migration patterns on the vessels, which is discussed in more detail below (Fantin et al. 2010; Smolders et al. 2017). Additionally, juxtavascular microglia migration has been described in the adult CNS, however this was in the context of an injury (Grossmann et al. 2002). If microglia migrate on vessels and utilize vascular networks as a scaffold for colonization of the healthy, postnatal brain are open questions. In Chapter II, I demonstrate that microglia are highly motile on vessels

during early postnatal development, but not in adulthood. This developmental timing of juxtavascular microglia motility suggests that microglia migrate on a vascular scaffold to colonize the developing cortex. Understanding precisely how microglia migrate and colonize the brain at the correct time is critical given the important role microglia play in regulating synapse maturation and pruning during critical windows of development, which is disrupted in diseases such as ASDs (Paolicelli et al. 2011; Hoshiko et al. 2012; Tremblay, Lowery, and Majewska 2010; Schafer et al. 2012; Gunner et al. 2019; Edmonson, Ziats, and Rennert 2016; Morgan et al. 2010).

Juxtavascular microglia

Parenchymal microglia that interact with the brain vasculature were first identified in the rodent brain in 1991 (Ashwell 1991; Lassmann et al. 1991). However, since their initial description, the vast majority of studies assessing interactions between microglia and the vasculature are in the context of disease and in the embryonic brain. In this thesis, I report the first extensive analysis of juxtavascular microglia in the healthy, postnatal and adult brain. Given the importance of juxtavascular microglia in inflammation, neurodegenerative disease, and stroke (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018; Fantin et al. 2010), a greater understanding of microglia-vascular interactions under healthy, steady state conditions is necessary and may provide novel therapeutic targets to treat the diseased CNS.

Juxtavascular microglia in the diseased CNS

It has become increasingly appreciated that acute and chronic peripheral inflammations (e.g. changes in microbiome or infection) induce large-scale changes in microglia reactivity and BBB impairment, which negatively impact CNS function (Morris et al. 2018; Sousa et al. 2018; Hanamsagar and Bilbo 2017; Hammond, Robinton, and Stevens 2018; Zhao et al. 2018; Rothhammer et al. 2018). Activated microglia secrete a range of toxic molecules such as reactive oxygen species (ROS), nitric oxide (NO), prostaglandin E2 (PGE), cyclooxygenase (COX)-2, quinolinic acid, several chemokines such as monocyte chemoattractant protein 1 (MCP-1), CXCL-1, and macrophage inflammatory protein (MIP)-1 α , and pro-inflammatory cytokines such as IL-6, tumor necrosis factor alpha (TNF- α), and IL-1 β , all of which exert a detrimental effect on the integrity and function of the BBB (Morris et al. 2018; Sousa et al. 2018). Additionally, *in vivo* 2-photon live imaging demonstrates that systemic inflammation induces a significant increase in the number of juxtavascular microglia just one day after infection (Haruwaka et al. 2019). These juxtavascular microglia initially maintain BBB integrity via expression of the tight junction protein Claudin-5, however during sustained inflammation, microglia phagocytose astrocytic endfeet and impair BBB function (Haruwaka et al. 2019). With new evidence that microglia could be a conduit between peripheral immunity and the CNS, these studies raise the interesting possibility that juxtavascular microglia are first responders to peripheral immune challenge and

perpetuate CNS inflammation by impairing BBB function (Hanamsagar and Bilbo 2017; Hammond, Robinton, and Stevens 2018; Zhao et al. 2018; Rothhammer et al. 2018).

In addition to systemic inflammation, several neurodegenerative diseases, such as Alzheimer's disease (AD) and Multiple sclerosis (MS), are characterized by vascular dysfunction and BBB breakdown that is attributed to an accumulation of juxtavascular microglia (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018). In AD, β -amyloid ($A\beta$) plaques are frequently found on blood vessels, with microglia highly localized around the perivascular deposits (Hickman and El Khoury 2010; Stankovic, Teodorczyk, and Ploen 2016). These juxtavascular microglia evoke an opening of the BBB through release of ROS and MMPs, causing endothelial cell damage and infiltration of peripheral immune cells into the CNS, perpetuating disease progression (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018; Morris et al. 2018). In MS, histopathological analyses of pre-demyelinating lesions has identified BBB disruption and activated microglia as the earliest pathological signs of disease (Marik et al. 2007). In fact, juxtavascular microglia accumulate on blood vessels prior to myelin loss or paralysis onset in a rodent model of MS (Experimental Autoimmune Encephalomyelitis (EAE)) through the interaction of CD11b on microglia and the blood component fibrinogen (Adams et al. 2007; Davalos et al. 2012). Hindering the fibrinogen–juxtavascular microglia interaction by pharmacological and genetic means led to reduced severity of EAE, suggesting that juxtavascular microglia

contribute to disease progression (Adams et al. 2007; Davalos et al. 2012). Further, these fibrinogen induced juxtavascular microglia generate elevated ROS, which causes BBB breakdown and peripheral immune cell infiltration (Davalos et al. 2012; Stankovic, Teodorczyk, and Ploen 2016; Morris et al. 2018).

Finally, stroke initiates robust microglial inflammatory response and BBB breakdown (Gelderblom et al. 2009; Schilling et al. 2003). The initial breakdown of the BBB after stroke causes release of blood proteins such as fibrinogen and albumin into the brain parenchyma, which attract microglia to become juxtavascular 24 hours after ischemic insult (Jolivel et al. 2015). Once juxtavascular, microglia disrupt BBB integrity by phagocytizing endothelial cells, generating ROS, releasing MMPs, and producing pro-inflammatory cytokines (Jolivel et al. 2015; Stankovic, Teodorczyk, and Ploen 2016). Interestingly, data suggests that inhibiting microglia during the first 24 hours after ischemic stroke may reduce BBB damage and facilitate recovery of the ischemic penumbra. For example, inhibiting microglial activation, by treating animals with minocycline, decreased the secretion of IL-1 β and NO in microglia, which correlated with a smaller infarct size (Yenari et al. 2006; Yrkanheikki et al. 1999). Whether specifically inhibiting juxtavascular microglia improves recovery after stroke is unknown.

Microglial interactions with the vasculature play a clear role in disease progression, but surprisingly little is known about these interactions in the healthy developing and adult brain. A deeper understanding of precisely when, where,

and mechanisms by which microglia interact with the vasculature in the healthy brain may lead to novel therapeutic strategies to reduce vascular pathology and facilitate recovery of neurological disorders. In this thesis, I determine that microglia directly contact the vasculature during early postnatal development through adulthood, filling a significant gap in knowledge in the field. Moreover, I demonstrate that CX3CR1 regulates the timing of these interactions in the developing brain, enabling future investigation of CX3CR1 as a potential therapeutic target to inhibit microglial localization to the vasculature in the diseased CNS.

Juxtavascular microglia in embryonic development

In developing mouse embryos, microglia invade the CNS as it is being vascularized, putting microglia in a unique position to influence the early sprouting, migration, anastomosis, and refinement of the growing CNS vascular system. Indeed, studies have shown that microglia associate with the vasculature during early embryonic development in both rodents and humans (Monier et al. 2007; Fantin et al. 2010; Smolders et al. 2017; Checchin et al. 2006; Rymo et al. 2011; Yoshiaki Kubota et al. 2009; Dudiki et al. 2020). These juxtavascular microglia migrate on vessels in the embryonic mouse brain, however the precise function of this migration is unknown. In addition to associating with vessels, it has been suggested that embryonic juxtavascular microglia regulate vascular complexity (Fantin et al. 2010; Smolders et al. 2017; Checchin et al. 2006; Rymo

et al. 2011; Yoshiaki Kubota et al. 2009; Dudiki et al. 2020). For example, in the embryonic brain, microglia are often localized to vascular junction points and depletion of all microglia is associated with a decrease in vascular branch points (Fantin et al. 2010). Similar findings have been identified in the developing retina (Rymo et al. 2011; Checchin et al. 2006; Dudiki et al. 2020). Several signaling mechanisms have been suggested to regulate this microglia-dependent vascular architecture, including Wnt-Flt1 and transforming growth factor β (TGF- β) 1 signaling, however these mechanisms were identified in the retina and have not been explored in the brain (Stefater et al. 2011; Dudiki et al. 2020).

These studies provide evidence that microglia interact with the vasculature in the embryonic brain; however, what happens to these juxtavascular microglia after embryonic development is not known. In Chapter II, I demonstrate that microglia are localized to the vasculature during postnatal development and are developmentally regulated, with a high percentage of microglia associated with the vasculature during the first week of postnatal development, which diminishes and then is maintained in adult animals. Moreover, I determine that microglia continue to be migratory on the vasculature during early postnatal development, however this motility is restricted in adulthood. This work addresses a critical need to understand juxtavascular microglia, an understudied subpopulation of microglia in the healthy, postnatal CNS.

The fractalkine receptor

Microglia, born in the embryonic yolk sac, invade the developing neuroepithelium at E9.5 where they are found associated with the vasculature (Fantin et al. 2010; Smolders et al. 2017; Ginhoux et al. 2010). In this thesis, I present evidence that microglia remain highly juxtavascular during the first week of postnatal development and this association diminishes as the rodent develops. However, the molecular mechanism regulating the developmental timing of microglia-vascular interactions remains unknown. An intriguing candidate molecule to regulate the timing of these interactions is CX3CR1, a 7 transmembrane domain G-protein-coupled chemokine receptor that is predominantly expressed in microglia in the CNS, as it has been implicated in regulating microglial recruitment and colonization (Combadiere, Ahuja, and Murphy 1995; Raport et al. 1995; Hoshiko et al. 2012; Paolicelli et al. 2011; Zhan et al. 2014). Mice deficient in CX3CR1 signaling have a delay in localization of microglia to synapse-dense regions of the hippocampus and somatosensory cortex, which is concomitant with a delay in synapse development (Paolicelli et al. 2011; Hoshiko et al. 2012; Zhan et al. 2014). Given that it is unknown how CX3CR1 signaling regulates microglial function, in this thesis CX3CR1 signaling is broadly defined as encompassing signaling through a down stream GPCR pathways, an extracellular domain function such as an adhesion molecule, or a scaffold/heterodimer protein-protein interaction with a second receptor. Despite growing evidence that CX3CR1 is important for proper neural circuit

development, the mechanism by which CX3CR1 regulates microglial recruitment to synaptic regions in the developing brain remains an open question. One possible mechanism by which CX3CR1 could regulate microglial recruitment is via vascular networks, as CX3CR1 has been implicated in regulating interactions between immune cells and the vasculature in the periphery (Fong et al. 1998; Hamon et al. 2017; Schwarz et al. 2010; Imai et al. 1997; Imaizumi, Yoshida, and Satoh 2004; Muehlhoefer et al. 2000; Umehara et al. 2004; Johnson and Jackson 2013; Goda et al. 2000). In Chapters II and III, I provide evidence that the timing of microglia-vascular interactions during development is regulated by CX3CR1 and suggest that CX3CR1-dependent microglia-vascular interactions are critical for microglial colonization to synapse-dense brain regions. This work provides insight into molecular mechanisms regulating microglial localization to the appropriate brain region at the correct time, an open question in the field.

The fractalkine receptor, microglial recruitment, and neuronal circuit development

CX3CR1 has been shown to regulate microglia colonization of cortical and subcortical brain regions, as well as subsequent synapse development. One such cortical region is the barrel cortex, which contains a highly precise synaptic map of the vibrissae (whiskers) on the snout. Sensory endings from trigeminal neurons transmit sensory information from each whisker follicle across several synapses, ultimately terminating at thalamocortical synapses within layer IV of the barrel cortex. These layer IV thalamocortical synapses form discrete barrel

structures corresponding to each whisker, which are separated by septa where thalamocortical synapses are largely absent (Woolsey and Van der Loos 1970; Welker and Woolsey 1974). Studies have shown that microglia first localize to the septa and then colonize these thalamocortical synapse-dense barrel centers between P6 and P7 and this timing is delayed to P8-P9 in CX3CR1 deficient (*Cx3cr1*^{-/-}) mice, without any changes in overall microglial density between control and *Cx3cr1*^{-/-} mice (Hoshiko et al. 2012). Importantly, this delay in recruitment is concomitant with a delay in functional synapse maturation, as measured by NMDA receptor subunit composition (Hoshiko et al. 2012). Similar delays in microglial recruitment to synapses were also observed between P8 and P28 in the synapse-dense CA1 region in the hippocampus of *Cx3cr1*^{-/-} mice (Paolicelli et al. 2011). This delay in localization was accompanied by increased dendritic spine numbers and immature synapses in *Cx3cr1*^{-/-} mice (Paolicelli et al. 2011). Finally, microglial colonization of the motor cortex during early postnatal development has also been shown to be impaired in the absence of fractalkine signaling, resulting in microglial accumulation within subcortical white matter of *Cx3cr1*^{-/-} mice at P5 (Ueno et al. 2013). Long term, *Cx3cr1*^{-/-} mice have deficits in functional connectivity, as well as ASDs-like behaviors including defects in social interactions and increased repetitive behaviors (Zhan et al. 2014). These data raise the intriguing possibility that the fine-scale timing of microglial cells to specific brain regions is critical for neural circuit development relevant to ASDs. Despite growing evidence that CX3CR1 is important for proper

neural circuit development, the mechanism by which CX3CR1 regulates microglial recruitment to synaptic regions in the developing brain remains an open question.

Fractalkine signaling in immune cell adhesion to endothelial cells

In the periphery, circulating immune cells, such as leukocytes or monocytes, associate with blood vessels when they extravasate across the walls of microvessels into peripheral tissues. The classical pathway of leukocyte migration begins with selectin-mediated interactions between rolling leukocytes and the endothelium, decelerating the flowing leukocytes (B. Jones, Beamer, and Ahmed 2010). Locally produced chemokines from the tissue activate integrins on leukocytes (such as CD11a, CD11b, or CD11c) and intracellular adhesion molecules (including intracellular adhesion molecule (ICAM)-1/ICAM-2 and members of the junctional adhesion molecules family) that coat the endothelium, resulting in a strong adhesion between the leukocyte and endothelial cell (Oda et al. 1992; B. Jones, Beamer, and Ahmed 2010). After adhesion, transendothelial migration occurs by paracellular trafficking between the endothelial cells or intracellular trafficking through endothelial cytoplasmic pores (Oda et al. 1992; B. Jones, Beamer, and Ahmed 2010).

Prior to the identification and description of fractalkine (CX3CL1), the only known *in vivo* ligand to CX3CR1, it was thought that all chemokines that attracted leukocytes to the endothelium were soluble molecules that associated with cell

surface proteoglycans and tissue matrix components to retain the local chemokine gradient (Imai et al. 1997; Imaizumi, Yoshida, and Satoh 2004; Umehara et al. 2004; Tanaka et al. 1993). However, CX3CL1 is unique amongst chemokines because it is synthesized as a transmembrane molecule consisting of an extracellular N-terminal chemokine domain, a mucin-like stalk, a transmembrane α helix, and a short cytoplasmic tail (Umehara et al. 2004). Therefore, CX3CL1 itself functions as an adhesion molecule, enhancing the capture, adhesion, and subsequent migration of immune cells to the vasculature (Bazan et al. 1997; Imai et al. 1997). Indeed, CX3CR1-expressing cells bind rapidly with high affinity to immobilized CX3CL1 or CX3CL1-expressing cells in both static and physiological flow conditions (Imai et al. 1997; Goda et al. 2000; Fong et al. 1998). Thus, CX3CL1 may facilitate the extravasation of circulating leukocytes by mediating cell adhesion through the initial tethering and final transmigration steps (Umehara et al. 2004). In addition to CX3CL1 adhesion, CX3CL1-CX3CR1 can transduce signals through G proteins that enhance leukocyte integrin binding to its ligands (Goda et al. 2000). Therefore, the engagement of both CX3CR1 on leukocytes with CX3CL1 and intracellular adhesion molecules on endothelial cells results in enhanced cell adhesion compared to each system alone (Umehara, Bloom, et al. 2001; Umehara, Goda, et al. 2001).

These data demonstrate that CX3CR1 signaling is important for the proper timing of microglial colonization of cortical and subcortical brain regions, as well

as the adhesion of peripheral immune cells to the vasculature, raising the intriguing possibility that CX3CR1 signaling may regulate the timing of microglia-vascular interactions in the developing brain. In Chapter II, I demonstrate that the timing of juxtavascular microglia is developmentally delayed in CX3CR1 deficient mice. This delay in association coincides with a delay in microglial localization into barrel centers in CX3CR1 deficient mice, suggesting that microglia-vascular interactions are important for the proper colonization of synapse-dense brain regions. In Chapter III, I demonstrate that the CX3CR1-dependent delay in juxtavascular microglia is independent of CX3CL1, the only known *in vivo* ligand for CX3CR1, suggesting that delays in the timing of juxtavascular microglia in CX3CR1 deficient mice are not due to direct adhesion between CX3CL1-CX3CR1. Understanding the molecular mechanism underlying the timing of microglia colonization to synapse-dense brain regions may provide important insight into neurodevelopmental disorders, such as ASDs, where changes in microglial numbers and synaptic connectivity have been implicated in disease progression (Edmonson, Ziats, and Rennert 2016; Morgan et al. 2010).

Thesis Overview

Together, my thesis work aims to understand juxtavascular microglia in the healthy, developing and adult CNS. While microglial interactions with neurons, synapses, and other glial cells are well understood, far less is known about microglial interactions with the CNS vasculature. Questions include: What

is the developmental timing of microglial interactions with the vasculature in the healthy CNS? Where within the cellular makeup of the NVU do microglia reside? Are microglia stable or dynamic while associated with the vasculature? What molecular mechanism regulates the timing of microglia vascular interactions? Answers to these questions will provide insight into juxtavascular microglia, a subpopulation of microglia that has never been studied in the healthy postnatal CNS, as well as neurological diseases where juxtavascular microglia contribute to vascular dysfunction and disease progression.

In Chapter II, I address the developmental timing, dynamics, and cellular localization of juxtavascular microglia in the healthy brain. My results indicate that a high percentage of microglia are associated with the vasculature during the first week of postnatal development and this timing is dependent on CX3CR1. Moreover, microglia-vascular interactions are concomitant with a peak in microglial colonization of the cortex and recruitment to synapses. I find that as microglia colonize the cortex, vascular-associated microglia are highly motile along vessels and become largely stationary as astrocyte endfeet arrive and the brain matures in adult mice. Finally, my work demonstrates that juxtavascular microglia associate with both the basal lamina and astrocyte endfeet surrounding the vasculature through development and adulthood. These findings lay the fundamental groundwork to investigate juxtavascular microglia function, microglia-astrocyte crosstalk, and microglia-vascular crosstalk in the healthy and diseased brain. They further provide a potential mechanism by which vascular

interactions facilitate microglial colonization of the brain to later regulate neural circuit development.

In Chapter III, I investigate the molecular mechanisms underlying the timing of microglia-vascular interactions in the developing brain. I find that CX3CR1 regulates the timing of microglia-vascular interactions across cortical brain regions. Further, I show that CX3CR1 facilitates microglial adhesion to endothelial cells in young postnatal mice, a time of active microglial colonization, but not older mice. Finally, I demonstrate that the timing of microglia-vascular interactions in the developing brain is independent of the canonical CX3CR1 ligand, fractalkine (CX3CL1). These data provide the first mechanistic insight into the CX3CR1-dependent timing of microglia-vascular interactions in the postnatal brain. Altogether, these studies presented in this dissertation aim to advance our understanding juxtavascular microglia in the healthy, developing and adult brain.

CHAPTER II

A developmental analysis of juxtavascular microglia dynamics and interactions with the vasculature

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Contribution Summary

E.M. collected confocal microscopy, structured illumination microscopy, expansion microscopy, acute slice culture live imaging, 2-photon live imaging images and quantified all results. S.C.B. quantified juxtavascular microglia that occur by chance and juxtavascular microglia on branched and unbranched segments. A.G.K. quantified juxtavascular microglia in WT animals. M. Schifferer collected electron microscopy images and 3D reconstructions. C.E.B. assisted with structured illumination microscopy. J.C. imaged and analyzed human juxtavascular microglia. E.J.H, M. Simons, and D.P.S. designed research. E.M. and D.P.S. designed and interpreted all experiments and wrote the final document.

ABSTRACT

Microglia, the resident macrophages of the central nervous system (CNS), are dynamic cells, constantly extending and retracting their processes as they contact and functionally regulate neurons and other glial cells. There is far less known about microglia-vascular interactions, particularly under healthy steady-state conditions. Here, we use the male and female mouse cerebral cortex to show that a higher percentage of microglia associate with the vasculature during the first week of postnatal development compared to older ages and the timing of these associations are dependent on the fractalkine receptor (CX3CR1). Similar developmental microglia-vascular associations were detected in the prenatal human brain. Using live imaging in mice, we found that juxtavascular microglia migrated when microglia are actively colonizing the cortex and became stationary by adulthood to occupy the same vascular space for nearly 2 months. Further, juxtavascular microglia at all ages associate with vascular areas void of astrocyte endfeet and the developmental shift in microglial migratory behavior along vessels corresponded to when astrocyte endfeet more fully ensheath vessels. Together, our data provide a comprehensive assessment of microglia-vascular interactions. They support a mechanism by which microglia use the vasculature to migrate within the developing brain parenchyma. This migration becomes restricted upon the arrival of astrocyte endfeet such that juxtavascular microglia become highly stationary and stable in the mature cortex.

INTRODUCTION

While myeloid lineage in origin, microglia are now appreciated to be key cellular components of neural circuits. Imaging studies have revealed that microglia are constantly extending and retracting their processes, which are in frequent contact with neurons, synapses, and other glial cells (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005; Schafer et al. 2012; Tremblay, Lowery, and Majewska 2010; Frost and Schafer 2016). These descriptions of interactions between microglia and other resident CNS cell types have now led to a new understanding that microglia are important for neural circuit structure and function, including their role in developmental synaptic pruning by engulfing and removing synapses from less active neurons (Schafer et al. 2012; Tremblay, Lowery, and Majewska 2010; Paolicelli et al. 2011; Gunner et al. 2019). Besides interactions with parenchymal neurons and glia, microglia are known to interact with the vasculature. However, the vast majority of these studies have been in the context of disease where parenchymal microglia rapidly associate with the brain vasculature following breakdown of the blood-brain barrier (BBB) and, in turn, inflammatory microglia can modulate the breakdown of the BBB (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018). Far less is known about how microglia interact with the vasculature in the healthy brain. With new evidence that microglia could be a conduit by which changes in peripheral immunity (e.g. microbiome, infection, etc.) affect CNS function (Hanamsagar and Bilbo 2017; Hammond, Robinton, and Stevens 2018; Zhao et

al. 2018; Rothhammer et al. 2018) and mounting evidence that an array of neurological disorders have a vascular and microglial component (Daneman 2012; Hammond, Robinton, and Stevens 2018; Zhao et al. 2018), a greater understanding of microglia-vascular interactions is necessary.

The neurovascular unit (NVU) is composed of endothelial cells, pericytes, vascular smooth muscle cells, astrocytes, macrophages, and neurons that connect the brain parenchyma to the cerebral vasculature. Interactions between these NVU cell types is important for a variety of physiological processes such as angiogenesis, vessel maintenance and permeability, metabolic support, and regulation of blood flow (L. S. Brown et al. 2019; McConnell et al. 2017). The development of the NVU begins around embryonic day (E) 9.5 in mice, when specialized endothelial cells branch from vessels of the perineural vascular plexus to form capillaries that invade nearby neural tissue (Saili et al. 2017). Pericytes associate with endothelial cells as nascent vessels generate at E9.5 (Armulik et al. 2010; Bauer et al. 1993; Yamanishi et al. 2012; Daneman et al. 2010) and these interactions are critical to form the BBB (Zlokovic 2008; Daneman et al. 2010). Astrocytes are also a key component of the mature NVU. After the vasculature initially forms, astrocytes extend their processes to form endfeet over the course of postnatal development in rodents (Daneman et al. 2010). These astrocyte endfeet ultimately surround and ensheath the majority of the vasculature by adulthood where they play roles in a variety of functions such as maintaining the BBB, providing metabolic support to neurons, and regulating

blood flow (Abbott, Rönnbäck, and Hansson 2006; Kimelberg and Nedergaard 2010; Macvicar and Newman 2015).

The vast majority of studies assessing interactions between microglia and the vasculature are in the context of disease. For example, microglia rapidly surround and contact the vasculature following breakdown of the BBB in the inflamed CNS (Zhao et al. 2018; Stankovic, Teodorczyk, and Ploen 2016). One mechanism regulating these microglia-vascular interactions is the blood component fibrinogen and CD11b on microglia (Davalos et al. 2012; Adams et al. 2007). Reactive microglia can also influence the opening of the BBB by phagocytosing astrocyte endfeet or upregulating molecules such as vascular endothelial growth factor (VEGF), inducible nitric oxide synthase (iNOS), and reactive oxygen species (ROS) (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018; Haruwaka et al. 2019). In the healthy brain, much less is known. Studies in rodents and humans have shown that microglia associate with the vasculature in the developing CNS and live imaging in postnatal brain slices following traumatic injury or in embryonic mouse brain slices has suggested that microglia can migrate along the vasculature (Monier et al. 2007; Fantin et al. 2010; Smolders et al. 2017; Grossmann et al. 2002; Checchin et al. 2006). Microglia have also been suggested to regulate vascular growth and complexity in the developing hindbrain and retina (Fantin et al. 2010; Rymo et al. 2011; Checchin et al. 2006; Yoshiaki Kubota et al. 2009; Dudiki et al. 2020). Together,

these studies provide evidence that there is microglia-vascular crosstalk, which requires further investigation in development, adulthood, and disease.

In the current study, we investigated microglia-vascular interactions in the healthy, developing and adult cerebral cortex. Using confocal, super-resolution, expansion, and electron microscopy, we assessed the developmental regulation of associations between microglia and the vasculature and used fractalkine receptor (CX3CR1)-deficient mice to determine a role for this signaling in the timing of these interactions. Using *in situ* confocal and *in vivo* 2-photon live imaging, we further assessed the dynamics of juxtavascular microglia in real time. Our data support a mechanism by which microglia migrate along the vasculature to colonize the developing brain and the timing of these interactions is regulated by CX3CR1. This migratory behavior becomes restricted as astrocyte endfeet mature and suggests the establishment of a long-term niche for juxtavascular microglia in the adult brain.

RESULTS

A high percentage of microglia are juxtavascular during development

During rodent and human embryonic development, microglia somas have been described to be in close association with blood vessels (i.e. juxtavascular microglia) (Fantin et al. 2010; Monier et al. 2007; Checchin et al. 2006). We assessed microglial association with the vasculature over an extended developmental time course across postnatal development. Microglia were labeled using transgenic mice that express EGFP under the control of the fractalkine receptor CX3CR1 (*Cx3cr1^{EGFP/+}*). The vasculature was labeled with an antibody against platelet endothelial cell adhesion molecule (PECAM). To start, we focused our analyses in the frontal cortex. Juxtavascular microglia were defined as microglia with at least 30% of their soma perimeter in association with blood vessels and soma centers that were within 10 μ m of the vessel, which we confirmed with orthogonal views and 3D surface rendering (Fig 2.1 A-F; See also Movies 1 and 2). Juxtavascular microglia were further distinguished from perivascular macrophages by their morphology with processes emanating from their soma and higher levels of EGFP. Using these criteria, we found the percent of total microglia associated with vasculature and microglial soma surface area associated with the vessel was higher during early development than in older animals in the frontal cortex (Fig 2.1 G-H). We further found a higher percent of the total microglial population were juxtavascular at P1-P5 in the frontal cortex (Fig. 2.1 G). These results were independent of sex (data not shown). The

percent association dropped to below 20% by P14 and was maintained at later ages. We confirmed that this developmental regulation of juxtavascular microglia was independent of changes in vasculature density over development. While the total vascular content of the cortex increases as the brain grows, the density of the blood vessels within a given field of view is unchanged across development (Fig. 2.1 I). Moreover, developmental changes in juxtavascular microglia were likely not due to chance encounters with the vasculature, as rotating the blood vessel images 180° resulted in reduced percentages of association with the vasculature over development (Fig. 2.1 J). Consistent with the results in mouse, the ventricular and subventricular zones of the prenatal human brain at the level of the frontal cortex also showed a high percent of juxtavascular microglia. This association in the developing human brain peaked at 18-24 gestational weeks (GW) where 38% of total microglia were juxtavascular (Fig. 2.1 K-L)—a percentage similar to what we identified in early postnatal mice. Together, these data demonstrate that a large percentage of the total microglia are juxtavascular in the early postnatal mouse and prenatal human brain.

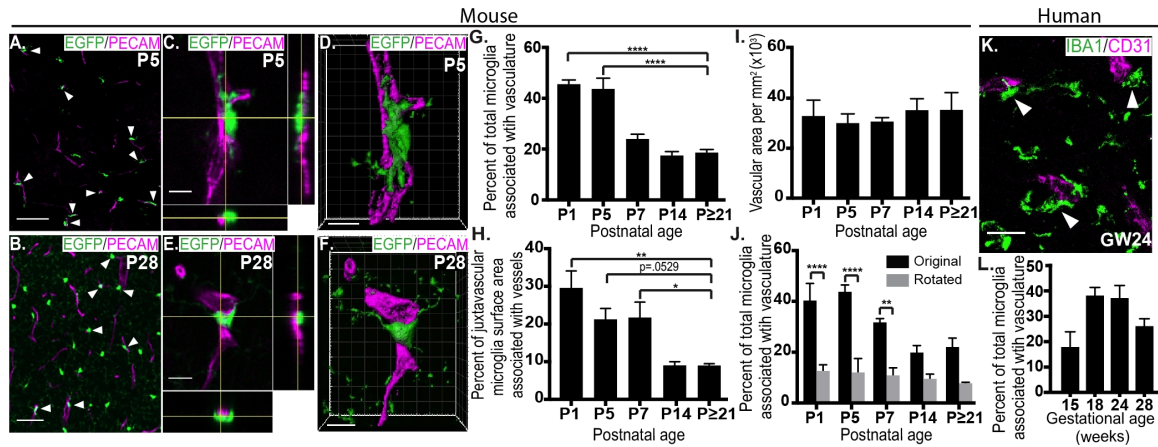


Figure 2.1: A high percentage of microglia are juxtavascular during early postnatal development. **A-B.** Representative low magnification tiled images of microglia (green, EGFP) associated with vasculature (magenta, anti-PECAM) in the P5 (**A**) and P28 (**B**) frontal cortex. Filled arrowheads denote juxtavascular microglia. Scale bars= 100 μ m (**A**) and 50 μ m (**B**). **C-D.** High magnification, orthogonal view (**C**) and 3D reconstruction and surface rendering (**D**) of juxtavascular microglia in the P5 frontal cortex (see also Movie 1). Scale bars= 10 μ m. **E-F.** Orthogonal (**E**) and 3D reconstruction and surface rendering (**F**) of a juxtavascular microglia in the P28 frontal cortex (see also Movie 2). Scale bars= 10 μ m. **G.** The percent of the total microglia population associated with vasculature over development in the frontal cortex. One-way ANOVA with Dunnett's post hoc; comparison to P \geq 21, n=4 littermates per developmental time point, ****p<.0001. **H.** Quantification of the percent of juxtavascular microglia surface area associating with vessels over development in the frontal cortex in 3D reconstructed confocal images. One-way ANOVA with Dunnett's post hoc; comparison to P \geq 21, n=3 littermates per developmental time point, *p= .0445, **p=.0025. **I.** Vascular density over development in the frontal cortex. One-way ANOVA with Dunnett's post hoc; comparison to P \geq 21, n=4 littermates per developmental time point. **J.** The percent of total microglia associated with vasculature that occur by chance in the frontal cortex over development. Two-way ANOVA with a Sidak's post hoc; n=4 littermates per developmental time point, **p=.0022, ****p<.0001. **K.** Representative image of microglia (green, anti-IBA1) associated with vasculature (magenta, anti-CD31) in gestational week (GW) 24 in the ventricular zone (VZ) and subventricular zone (SVZ) at the level of the human frontal cortex. Filled arrowheads denote juxtavascular microglia. Scale bar= 20 μ m. **L.** Quantification of the percentage of total microglia associated with vasculature in the human brain. One-way ANOVA across all ages, p=0.0544, n=1 specimen per gestational age. All error bars represent \pm SEM.

Juxtavascular microglia are largely associated with capillaries in the early postnatal cortex

While previous work has described similar high association of microglia with the vasculature in the embryonic/prenatal brain, these studies did not use markers to distinguish microglia from perivascular macrophages (Fantin et al. 2010; Monier et al. 2007; Checchin et al. 2006). Therefore, we next sought to confirm that vascular-associated EGFP-positive cells were, indeed, microglia versus perivascular macrophages and determine the types of vessel associated with juxtavascular microglia. We found that the juxtavascular EGFP+ cells that we initially identified as microglia based on their larger numbers of processes and higher levels of EGFP (Fig. 2.1; Fig. 2.2 A-B filled arrowheads) were also positive for the microglia-specific marker purinergic receptor P2Y12 (P2RY12) (Fig. 2.22 A, filled arrowhead) and negative for the perivascular macrophage-specific marker lymphatic vessel endothelial receptor 1 (LYVE1) (Fig. 2.2 B, unfilled arrowheads) (Butovsky et al. 2014; Zeisel et al. 2015). Using anti-P2RY12 to label microglia in wild-type mice or EGFP in *Cx3cr1*^{EGFP/+} mice, which are heterozygote for CX3CR1, we obtained similar percentages of juxtavascular microglial and vascular density (Fig. 2.2 C-D), confirming results were independent of the microglial labeling technique. We also found that these juxtavascular microglia were associated largely along unsegmented vessels, rather than branch points, across postnatal development (Fig. 2.2 E). We next assessed what types of vessels were associated with juxtavascular microglia,

using a combination of parameters. Capillaries are $\leq 8 \mu\text{m}$ in diameter and are smooth muscle actin (SMA)-negative and platelet derived growth factor receptor β (PDGFR β)-positive (Grant et al. 2019; Mastorakos and MCGavern 2019). Arterioles are $>8 \mu\text{m}$ in diameter and are SMA-positive and a subset of pre-capillary arterioles are also PDGFR β -positive (Grant et al. 2019). Using these markers, we identified that juxtavascular microglia were largely associated with capillaries ($\leq 8 \mu\text{m}$, SMA-negative, PDGFR β -positive; Fig. 2.2 F-H). These experiments establish that a large percentage of bona fide microglia are associated with unsegmented capillaries in the postnatal cerebral cortex and these percentages are similar in wild type and *Cx3cr1*^{EGFP/+} mice.

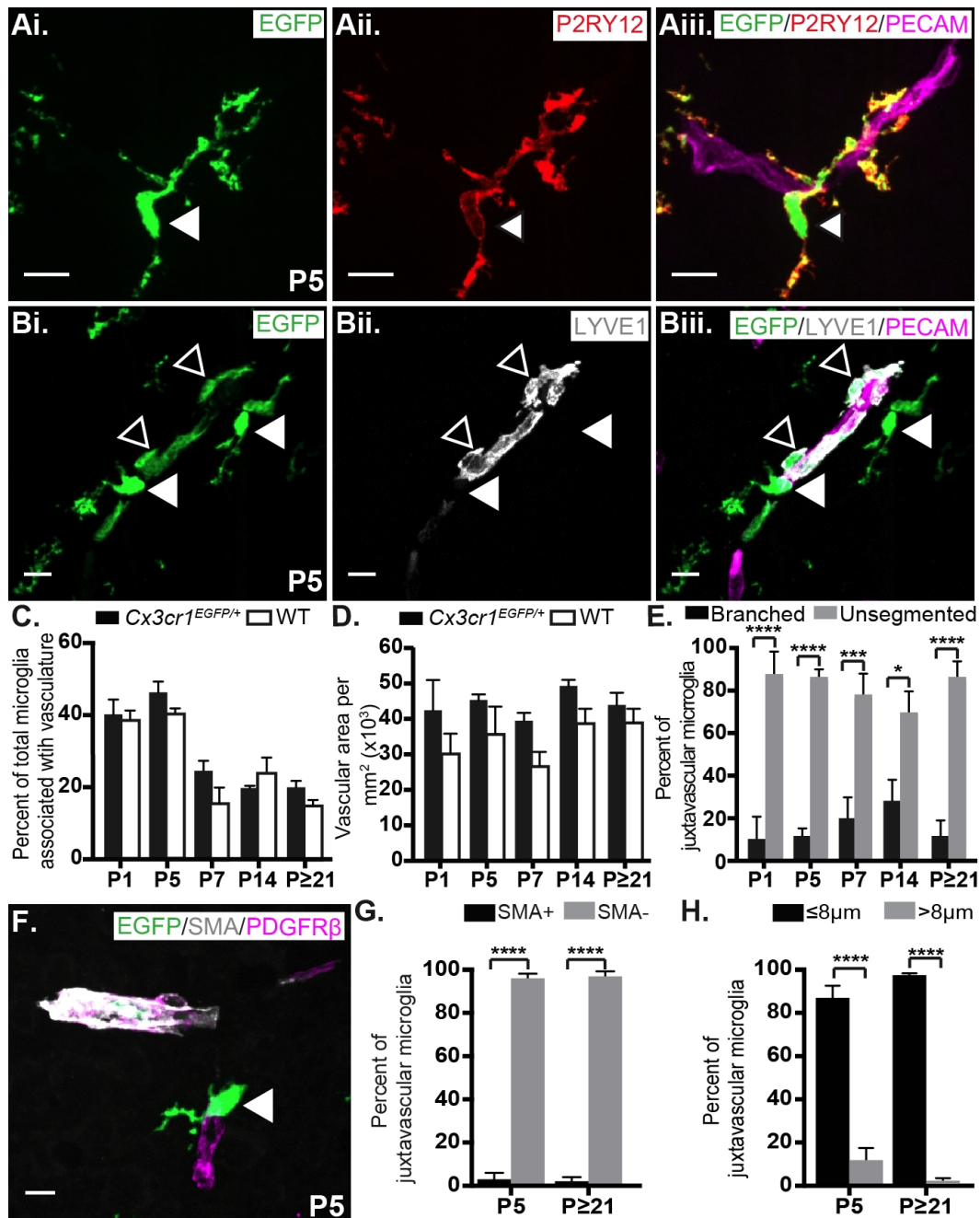


Figure 2.2: Juxtavascular microglia predominantly associate with capillaries in the postnatal cortex. **A.** A representative image of a juxtavascular microglia (filled arrowhead) in the P5 frontal cortex. Microglia are labeled using the *Cx3cr1*^{EGFP/+} reporter mouse (green; **Ai**) and immunolabeling for a microglia-specific marker anti-P2RY12 (red; **Aii**). The vasculature is labeled with anti-PECAM (magenta) in the merged image (**Aiii**). Scale bar= 10μm. **B.** A representative image of LYVE1-negative microglia (green, EGFP, filled

arrowheads) and LYVE1-positive perivascular macrophages (gray, anti-LYVE1, unfilled arrowheads) associated with vasculature (magenta, anti-PECAM) in the P5 frontal cortex. Scale bar= 10 μ m. **C.** Quantification of juxtavascular microglia across development labeled either with EGFP in *Cx3cr1*^{EGFP/+} mice (black bars) or anti-P2RY12 in wild type mice (WT, white bars) frontal cortices. Two-way ANOVA with a Sidak's post hoc; n=3-4 littermates per genotype per developmental time point. **D.** Quantification of vascular density in *Cx3cr1*^{EGFP/+} (black bars) and WT (white bars) frontal cortices over development. Two-way ANOVA with a Sidak's post hoc; n=3-4 littermates per genotype per developmental time point. **E.** Quantification of the percent of juxtavascular microglia associated with branched (black bars) or unsegmented (gray bars) vessels. Two-way ANOVA with a Sidak's post hoc; n=3-4 littermates per developmental time point, *p=.0118, ***p=.0003, ****p<.0001. **F.** A representative image of a juxtavascular microglia (green, EGFP, filled arrowhead) associated with smooth muscle cell actin (gray, SMA)-negative capillaries (magenta; PDGFR β) in the P5 frontal cortex. Scale bar= 10 μ m. **G.** Quantification of the percent of juxtavascular microglia associated with SMA-positive or -negative vessels at P5 and P \geq 21 in the frontal cortex. Two-way ANOVA with a Sidak's post hoc; n=3 littermates per genotype per developmental time point, ****p<.0001. **H.** Quantification of the percent of juxtavascular microglia associated with vessels \leq 8 μ m and $>$ 8 μ m at P5 and P \geq 21 in the frontal cortex. Two-way ANOVA with a Sidak's post hoc; n=4 littermates per genotype per developmental time point, ****p<.0001. All error bars represent \pm SEM.

High percentages of juxtavascular microglia occur when microglia are actively colonizing the cortex

Over development, microglia undergo a dynamic process of colonization and expansion in a rostral-to-caudal gradient (Ashwell 1991; Perry, Hume, and Gordon 1985). Similar to previously published work (Nikodemova et al. 2015), we identified a large expansion in cortical microglia between P1 and P14, with microglia colonizing the more rostral frontal cortex region prior to the more caudal somatosensory cortex (Fig. 2.3 A-C, bar graphs in B-C). Microglia-vascular association mirrored this rostral-to-caudal gradient by which microglia colonize the brain with a higher percentage of juxtavascular microglia at P1-P5 (46.3% at P1 and 44.4% at P5) in the frontal cortex and at P5-P7 (39.1% at P5 and 34.2% at P7) in the more caudal somatosensory cortex (Fig. 2.3 B-C, line graphs). Moreover, during times of active microglial colonization in both postnatal cortical regions (P1-P5 in the frontal cortex and P1-P7 in the somatosensory cortex), significantly more microglial primary processes were aligned parallel with vessels compared to older ages (Fig. 2.3 D-G). This parallel juxtavascular microglial orientation along vessels is consistent with a migratory orientation.

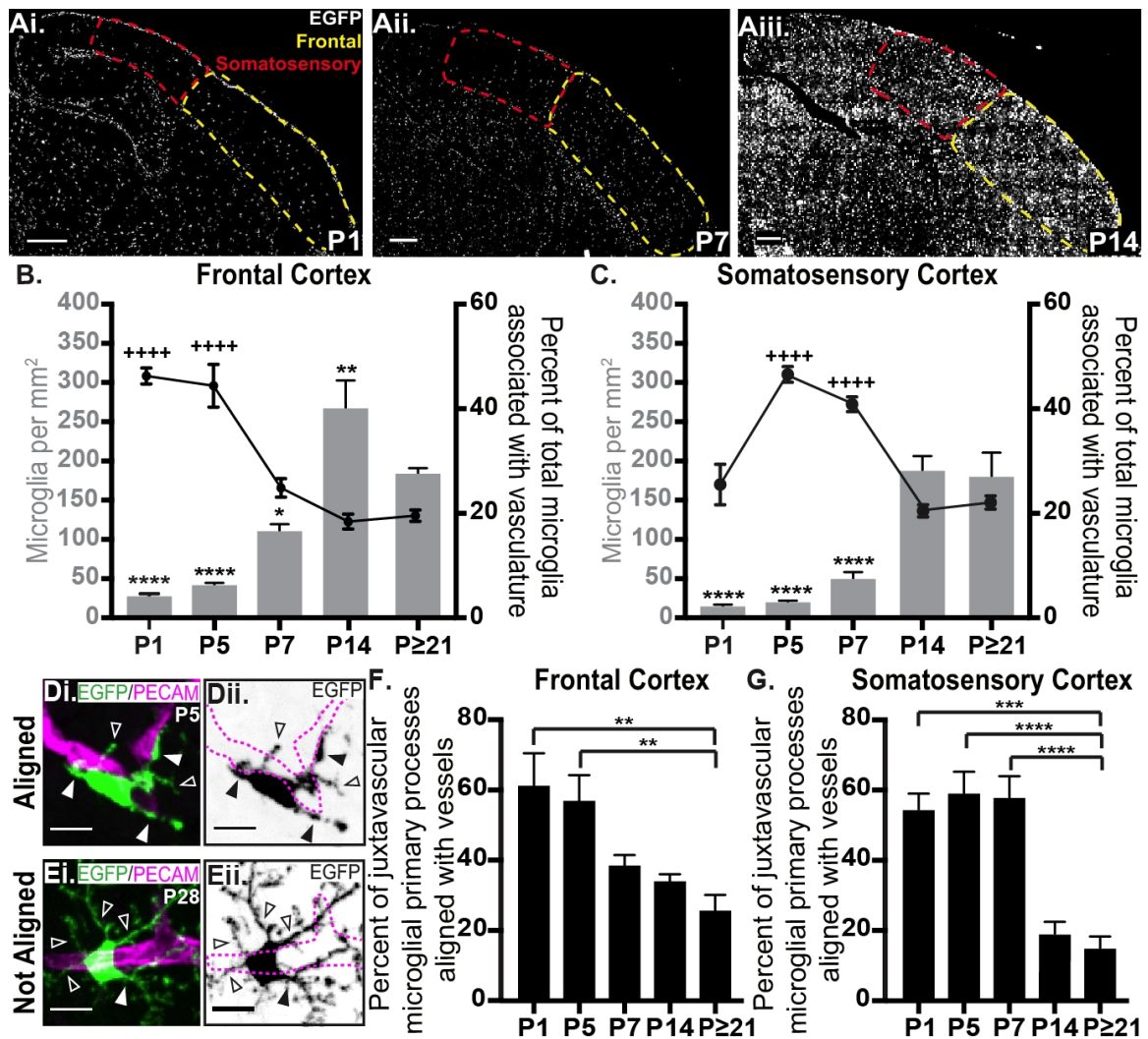


Figure 2.3: Microglia associate and align with vasculature as they colonize the cortex in a rostral-to-caudal gradient. **A.** Tiled sagittal sections of a P1 (**Ai**), P7 (**Aii**), and P14 (**Aiii**) *Cx3cr1^{EGFP/+}* brain. The dotted yellow and red lines outline the frontal and somatosensory cortex, respectively. Scale bars= 400 μ m. **B-C.** Left Y axis and gray bars: quantification of microglial density over development in the frontal cortex (**B**) and somatosensory cortex (**C**). One-way ANOVA with Dunnett's post hoc; comparison to P \geq 21, n=4 littermates per developmental time point, *p=.0182, **p=.0062, ****p<.0001. Right Y axis and black line graphs: the percent of the total microglia population associated with vasculature over development in the frontal cortex (**B**) and somatosensory cortex (**C**). Note, data corresponding to the percent of juxtavascular microglia in the frontal cortex (line graph in C) are the same as presented in Fig. 1G. One-way ANOVA with Dunnett's post hoc; comparison to P \geq 21, n=4 littermates per developmental time point, ****p<.0001. **D-E.** Representative images of

juxtavascular microglia (EGFP, green in **Di** and **Ei**; black in **Dii** and **Eii**) primary processes aligned parallel (**D**) with vessels (magenta, anti-PECAM) in the P5 frontal cortex, which were largely not aligned at P28 (**E**). Filled arrowheads denote processes aligned parallel to the vessel and unfilled arrowheads denote those microglial processes that are not aligned with the vessel. The dotted magenta line in **Dii** and **Eii** outline the vessel in **Di** and **Ei**. Scale bars= 10 μ m. **F-G**. Quantification of the percent of juxtavascular primary processes that are aligned parallel with vessels in the frontal (**F**) and somatosensory (**G**) cortices over development. One-way ANOVA with Dunnett's post hoc; comparison to P \geq 21, n=4 littermates per developmental time point, frontal cortex: **p=.0021 (P1), **p=.0033 (P5), somatosensory cortex: ***p=.0003, ****p<.0001). All error bars represent \pm SEM.

CX3CR1 regulates the timing of microglia-vascular interactions as microglia colonize synapse-dense cortical regions

To further investigate microglia-vascular interactions in the context of colonization of the postnatal cortex, we assessed a somatosensory sub-region where the pattern of microglial colonization has been well described—the barrel cortex. Layer IV of the barrel cortex contains thalamocortical synapses, which form a highly precise synaptic map of the vibrissae (whiskers) on the snout. These layer IV thalamocortical synapses form discrete barrel structures corresponding to each whisker, which are separated by septa where thalamocortical synapses are largely absent (Fig. 2.4 A) (Woolsey and Van der Loos 1970; Welker and Woolsey 1974). Previous work has shown that microglia first localize to the septa and then colonize these thalamocortical synapse-dense barrel centers between P6 and P7 and this process is delayed to P8-P9 day in CX3CR1-deficient (*Cx3cr1*^{-/-}) mice (Hoshiko et al. 2012). This delay in recruitment in *Cx3cr1*^{-/-} mice is concomitant with a delay in synapse maturation. However, it was unclear how CX3CR1 was regulating the timing of microglial recruitment to synapses in the barrel cortex. To identify barrels, we labeled thalamocortical presynaptic terminals with an antibody against vesicular glutamate transporter 2 (VGLUT2). Microglia were labeled with transgenic expression of EGFP in either *Cx3cr1*^{+/-} (*Cx3cr1*^{EGFP/+}) or *Cx3cr1*^{-/-} (*Cx3cr1*^{EGFP/EGFP}) mice. The vasculature was labeled with anti-PECAM. Similar to previous work (Hoshiko et al. 2012), microglia infiltrated thalamocortical synapse-

dense barrel centers (outlined with a yellow dotted line in Fig. 2.4 C-F) from the septa by P6-P7 in *Cx3cr1^{+/-}* mice and this process was delayed by one day in *Cx3cr1^{-/-}* mice (Fig. 2.4 B-D). Strikingly, just prior to entering barrel centers at P5-P6 in *Cx3cr1^{+/-}* mice, a higher percentage of microglia were juxtavascular (Fig. 2.4 E, G, arrowheads). Further, this microglia-vascular association was delayed by one day in *Cx3cr1^{-/-}* mice (Fig. 2.4 F-G), which is consistent with the delay in microglial migration into barrel centers in these mice (Fig. 2.4 B). In both genotypes, the percentage of juxtavascular microglia decreased once the microglia began to colonize the thalamocortical synapse-dense barrel centers, P7 in *Cx3cr1^{+/-}* mice and P8 in *Cx3cr1^{-/-}* mice (Fig. 2.4 E-G). These changes in microglia-vascular interactions were independent of any changes in total microglial or vascular density in layer IV (Fig. 2.4 H-I), but rather specific to microglial distribution between the septa and barrels. These data are consistent with a model by which microglia use the vasculature to colonize synapse-dense cortical regions at the appropriate developmental timing. They further suggest that CX3CR1 signaling modulates the timing of microglial-vascular interactions during colonization.

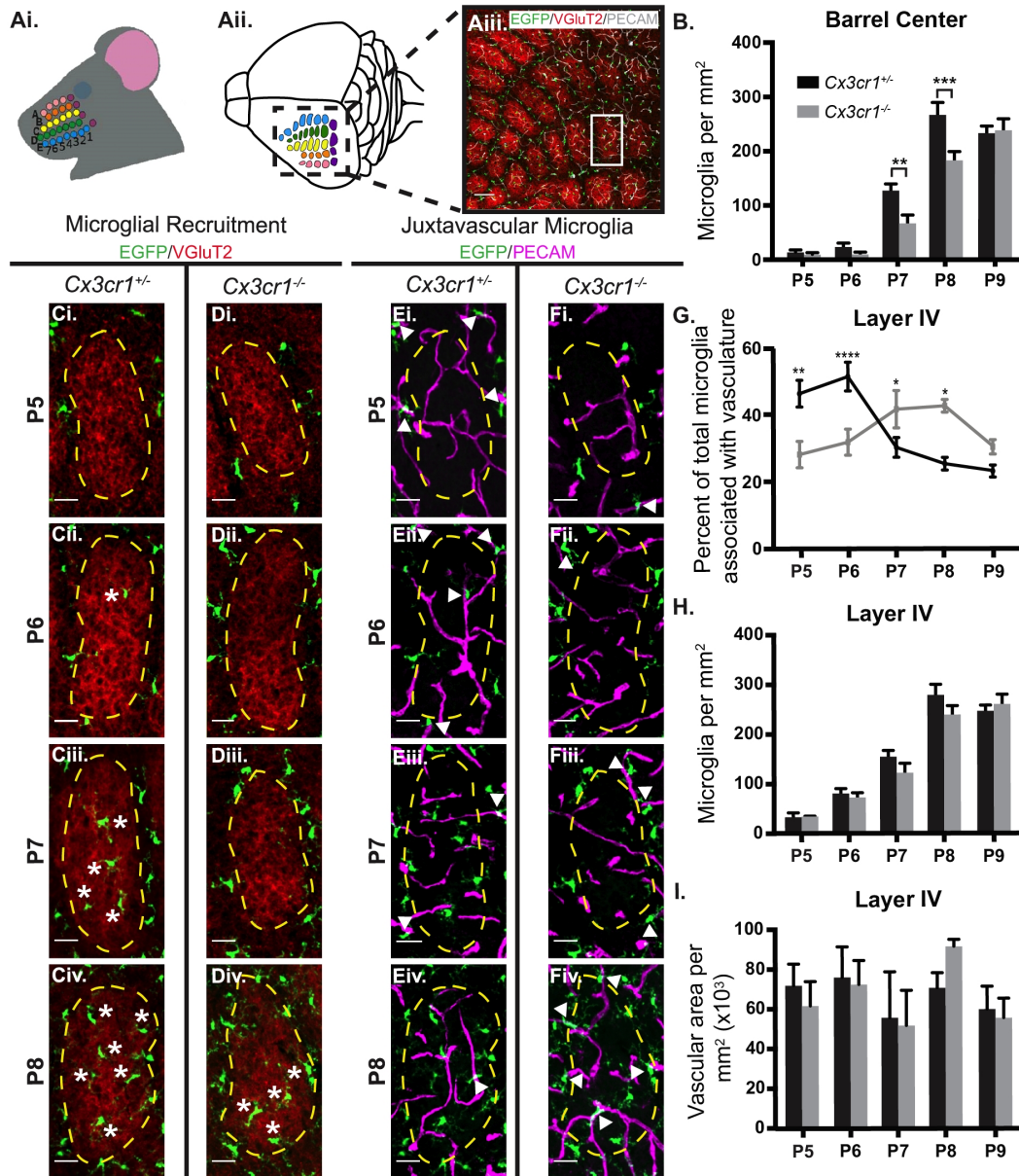


Figure 2.4: A high percentage of microglia associates with vasculature as they are recruited to synapses in the cortex and the timing is regulated by CX3CR1. Ai-Aii. Layer IV of the barrel cortex contains thalamocortical synapses, which form a highly precise synaptic map of the vibrissae (whiskers) on the snout. Aiii. A low magnification representative image of a tangential section through layer IV of the barrel cortex shows layer IV thalamocortical presynaptic terminals (red, anti-VGluT2), form discrete barrel structures corresponding to each whisker, which are separated by septa where thalamocortical terminals are

largely absent. Microglia are labeled by EGFP (green) and the vasculature is labeled with anti-PECAM (gray). White box denotes a single barrel. Scale bar= 100 μ m. **B.** Quantification of the number of microglia per mm² within the barrel centers in developing *Cx3cr1*^{+/-} (black bars) and *Cx3cr1*^{-/-} (gray bars) mice. Two-way ANOVA with a Sidak's post hoc; n=4 littermates per genotype per developmental time point; ** p=.0049, ***p=.0004. **C-D.** Representative images of quantification in B. Images are zoomed in to show single barrels within tangential sections of layer IV of the barrel cortex (denoted by white box in Aiii) where microglia (green) are recruited to barrel centers in *Cx3cr1*^{+/-} by P7 (**C**) and in *Cx3cr1*^{-/-} by P8 (**D**). Asterisks denote microglia located within barrel centers. The dotted yellow lines denote the perimeters of the VGluT2-positive thalamocortical inputs (red), which define the barrels vs. the septa. Scale bars= 30 μ m. **E-F.** The same representative fields of view in C-D but lacking the anti-VGluT2 channel and, instead, including the channel with anti-PECAM immunostaining (magenta) to label the vessels. Microglia are still labeled with EGFP (green). Dotted yellow lines still denote the perimeters of the VGluT2-positive barrels (red in C-D). Juxtavascular microglia in *Cx3cr1*^{+/-} (**E**) and *Cx3cr1*^{-/-} (**F**) mice are denoted by filled arrowheads. Scale bar= 30 μ m. **G.** Quantification of the percent of microglia associated with the vasculature in *Cx3cr1*^{+/-} (black lines) and *Cx3cr1*^{-/-} (gray lines) animals over development in layer IV of the barrel cortex demonstrates a peak of vascular association in *Cx3cr1*^{+/-} mice at P5-P6, which is delayed to P7-P8 in *Cx3cr1*^{-/-} coincident with delayed microglial recruitment to barrel centers. Two-way ANOVA with a Tukey's post hoc; n=4-5 littermates per genotype per developmental time point; *p=.0173 (P7), *p=.0187 (P8), **p=.0027, ****p<.0001, compared to P9 *Cx3cr1*^{+/-}. **H-I.** Quantification of microglial (**H**) and vascular (**I**) density in *Cx3cr1*^{+/-} (black bars) and *Cx3cr1*^{-/-} (gray bars) animals over development in layer IV of the barrel cortex. Two-way ANOVA with a Sidak's post hoc; n=4 littermates per genotype per developmental time point. All error bars represent \pm SEM.

Juxtavascular microglia migrate along the vasculature as they colonize the developing brain and are stationary in adulthood

With data demonstrating that high percentages of microglia are juxtavascular when they are actively colonizing the brain with processes aligned parallel to the vessel, we next performed live imaging to assess migration. As the early postnatal cortex is challenging to image *in vivo*, we performed our initial analyses in acute cortical slices. Acute slices of somatosensory cortex were prepared from early postnatal (P7) and adult (P \geq 120) *Cx3cr1^{EGFP/+}* mice, which were given a retro-orbital injection of Texas Red labeled dextran to label blood vessels prior to slice preparation. We then imaged microglia every 5 minutes over 6 hours at both ages (Fig. 2.5 A). Live imaging at P7 revealed significant juxtavascular microglial soma movement along blood vessels in the somatosensory cortex compared to vascular-unassociated microglia at P7 (Fig. 2.5 B, D, see also Movies 3-5). Specifically, 28.6% of juxtavascular microglia somas moved at a rate of 3-5 μ m/hour and another 26.1% moved at a rate of 5-7.5 μ m/hour (Fig. 2.5 D). In comparison, only 9.3% and 6.8% of vascular-unassociated microglia at the same age moved at 3-5 μ m/hour and 5-7.5 μ m/hour, respectively. We further found that when we assessed just the motile soma at P7, significantly more juxtavascular microglia somas travelled >20 μ m (30.9% traveled 20-30 μ m and 23.6% traveled 30-45 μ m) over 6 hours compared to vascular-unassociated microglia (7.5% and 6.8% traveled 20-30 μ m and 30-45 μ m, respectively) (Fig. 2.5 E). Importantly, the juxtavascular microglia soma

velocities and distances traveled are consistent with the rate and distances at which microglia migrate to barrel centers within the somatosensory cortex *in vivo* where the distance between the septa and barrel center is $\sim 80\mu\text{m}$ and it takes ~ 24 hours for microglia to reach the barrel center from the septa. Demonstrating directional motility and suggesting migration along the vessel, 84.1% of these postnatal juxtavascular microglia had a motility trajectory of $\leq 15^\circ$ along the blood vessel (Fig. 2.5 F). Together, these data demonstrate directional migration of juxtavascular microglia at distances and speeds consistent with colonization of the cortex (P7).

Interestingly, this migratory behavior along the vasculature was developmentally regulated and juxtavascular microglia in adult slices were largely stationary (Fig. 2.5 C-D; see also Movie 6). We further confirmed the stationary phenotype of juxtavascular microglia in the adult cortex by *in-vivo* 2-photon live imaging in *Cx3cr1^{EGFP/+}* mice. Windows were placed over the visual cortex, which was most conducive to our head posts necessary for stabilizing the head in awake, behaving mice during imaging. We have found similar microglia-vascular interactions by static confocal imaging in the visual cortex (data not shown). Mice were given a retro-orbital injection of Texas Red labeled dextran to label blood vessels prior to imaging and juxtavascular microglia were imaged every 5 min over the course of 2 hours (Fig. 2.5 G). As observed in acute cortical slices, 100% of juxtavascular and vascular-unassociated microglia were stationary (Fig. 2.5 H; see also Movie 7). To further understand long-term dynamics, we imaged

juxtavascular microglia *in vivo* over the course of 6 weeks (Fig. 2.5 I). We identified that 82.9% of juxtavascular microglia present on day 0 of imaging remained near the vasculature 6 weeks later (Fig. 2.5 J-K). Using static confocal imaging, we also found that juxtavascular microglia were less evenly distributed to their nearest neighbor compared to vascular-unassociated microglia, suggesting a preferential association with vessels in the adult vs. simply a consequence of tiling (Fig. 2.5 L). Together, these data demonstrate that juxtavascular microglia in the postnatal cortex are highly migratory compared to non-vascular associated microglia. In contrast, juxtavascular microglia in adulthood have a distribution that is less tiled compared to other parenchymal microglia and they are largely stationary, which suggests the establishment of a niche for juxtavascular microglia in the adult brain.

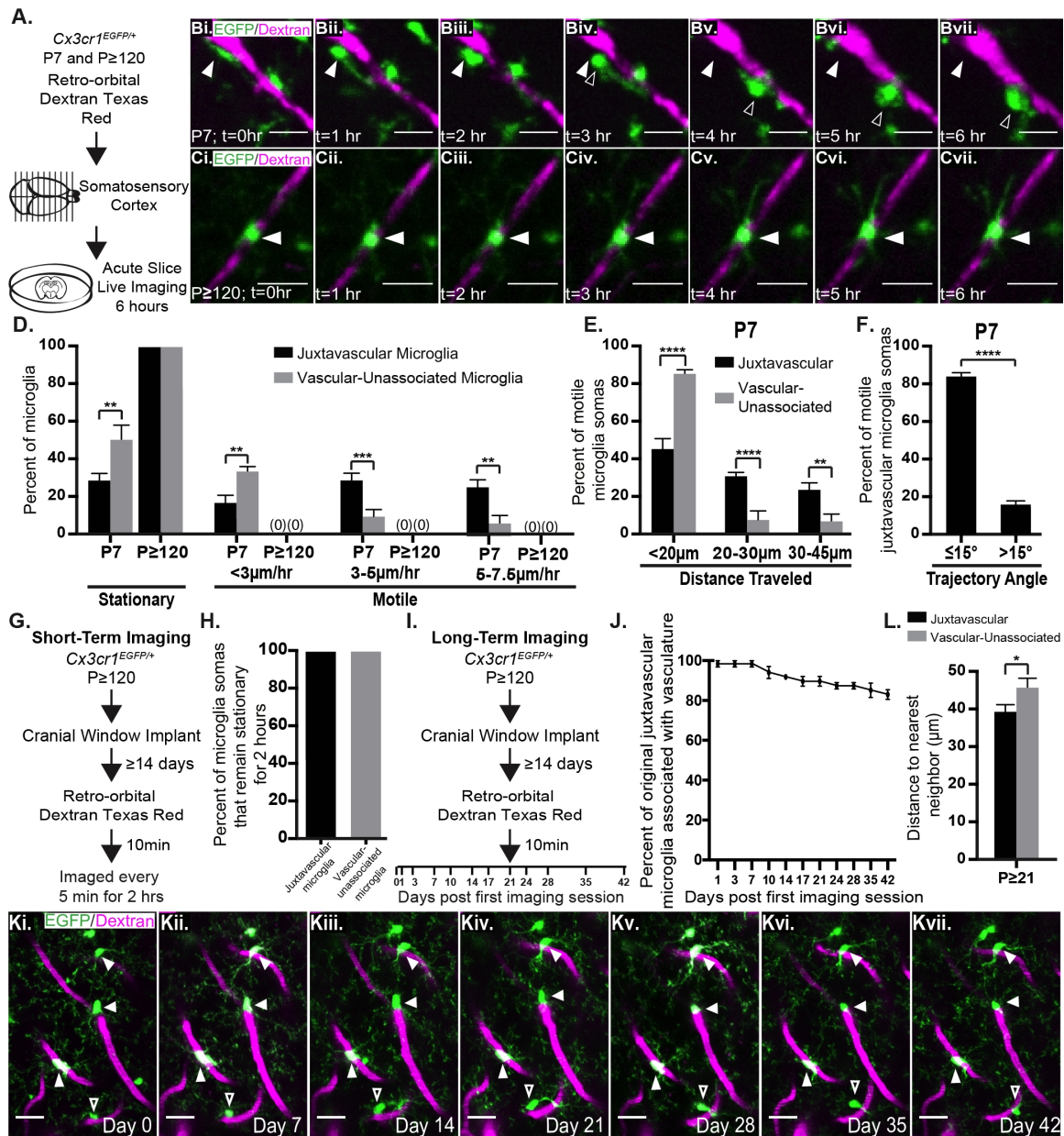


Figure 2.5: Juxtavascular microglia migrate along blood vessels as they colonize the developing brain and are largely stationary in adulthood. **A.** A schematic of the live imaging experiment. *Cx3cr1^{EGFP/+}* mice received a retro-orbital injection of Texas red labeled dextran to label the vasculature 10 minutes prior to euthanasia. Coronal somatosensory cortices were cut and imaged every 5 minutes over 6 hours immediately following slice preparation. **B-C.** Representative fluorescent images from a 6-hour live imaging session from a P7 (**B**) and P \geq 120 (**C**) slice. Filled arrowheads indicate microglial soma position at t=0. Unfilled arrowheads indicate the location of the same microglial soma at 0hr

(**Bi, Ci**), 1hrs (**Bii, Cii**), 2hrs (**Biii, Ciii**), 3hrs (**Biv, Civ**), 4hrs (**Bv, Cv**), 5hrs (**Bvi, Cvi**), and 6hrs (**Bvii, Cvii**). See also Movies 3-6. Scale bars= 30 μ m. **D.** Quantification of juxtavascular (black bars) and vascular-unassociated (gray bars) microglia soma motility speed/velocity. Two-way ANOVA with a Sidak's post hoc; n=4 mice per time point; **p=.0081 (stationary), **p=.0013 (0-3 μ m/hr), **p=.0015 (5-7.5 μ m/hr), ***p=.0005 (3-5 μ m/hr).. **E.** Quantification of the distance traveled of juxtavascular (black bars) and vascular-unassociated (gray bars) microglia somas in the P7 somatosensory cortex. Two-way ANOVA with a Sidak's post hoc; n=4 mice; **p=.0041, ****p<.0001. **F.** Quantification of migratory juxtavascular microglia trajectory angles in the P7 somatosensory cortex. Unpaired student's t-test; n=4 mice per time point; ****p<.0001. **G.** A schematic of short-term 2-photon live imaging experiment in adult cortex. *Cx3cr1*^{EGFP/+} mice received a retro-orbital injection of Texas Red-labeled dextran to visualize the vasculature 10 min prior to each imaging session. EGFP+ juxtavascular microglia were then imaged every 5 minutes for 2 hours. See also Movie 7. **H.** Quantification of the percent of juxtavascular (black bars) and vascular-unassociated (gray bars) microglia that remain stationary for 2 hours. Unpaired student's t-test; n=3 mice per developmental time point. **I.** A schematic of the long-term 2-photon live imaging experiment in adult visual cortex. *Cx3cr1*^{EGFP/+} mice received a retro-orbital injection of Texas Red-labeled dextran to visualize the vasculature 10 min prior to each imaging session. EGFP+ juxtavascular microglia were then imaged for 6 weeks. **J.** Quantification of the percent of juxtavascular microglia on vessels on day 0 that remain on vessels through six weeks of imaging. Data are representative of n=3 mice. **K.** Representative fluorescent images acquired during a 6-week live imaging session from a single mouse. Filled arrowheads indicate juxtavascular microglia that remain on vessels for 6 weeks. Unfilled arrowhead indicates a juxtavascular microglia that changes position, but remains on the vasculature, over 6 weeks. **L.** Quantification of the nearest neighbor distance between juxtavascular and vascular-unassociated microglia in static confocal images. Paired student's t-test; n=4 littermates, *p=0.0239. All error bars represent \pm SEM.

Microglia associate with the vasculature in areas lacking full astrocyte endfoot coverage

Our data demonstrate a strong microglial association and migration along the developing postnatal cortical vasculature. One possible mechanism regulating these developmental changes in juxtavascular microglia is the changing cellular composition of the NVU over development. The NVU begins to form during embryonic development, when pericytes associate with endothelial cells. Later in postnatal development, astrocytes are born and begin wrapping their endfeet around vessels until the vast majority of the vasculature is ensheathed by astrocyte endfeet by adulthood (Daneman et al. 2010; Schiweck, Eickholt, and Murk 2018; Bayraktar et al. 2015). As previously described (Daneman et al. 2010), the territory of Aquaporin 4 (AQP4)-positive astrocyte endfeet on PDGFR β + capillaries was low in the early postnatal cortex and then expanded over the first postnatal week (Fig. 2.6 A-D, bar graph in D). In more mature animals (\geq P21), astrocytic endfeet covered \sim 85% of vessels in the frontal cortex. Intriguingly, this developmental timing of astrocyte endfoot coverage mirrored the developmental shift in the percentage of juxtavascular microglia in the cortex (Fig. 2.6 D, line graph). That is, as the percentage of juxtavascular microglia decreased, astrocyte endfoot coverage increased. This astrocyte coverage also correlated with the timing of decreased microglial motility along the vessels (Fig. 2.5). We next assessed microglia-astrocyte endfoot interactions by confocal microscopy and 3D surface rendering at all ages and found that

microglia only associated with the vasculature in areas either completely void of astrocyte endfeet or in areas where vessels were not fully covered by the endfeet (Fig. 2.6 A-C, white arrow heads; Fig. 2.6 E, see also Movies 8-10). We also found microglia occupied 4-9% of the vessel area, depending on postnatal age, and there were parts of the vessel that lacked astrocyte endfeet that also lacked microglia (Fig. 2.6 F).

Given that cells of the NVU are nanometers apart from each other, we confirmed these results with expansion microscopy (ExM; Fig 2.6 G-H), structured illumination microscopy (SIM; Fig. 2.6 I-J) and electron microscopy (EM; Fig 2.7). By EM, microglia were identified based on characteristic microglial morphologies. Microglia nuclei tend to be half-mooned shape or long and thin with electron dense heterochromatin around the edge of the nucleus. Microglia were further distinguished by EM from perivascular macrophages by having processes emanating from the soma. Serial sectioning and 3D reconstruction of a representative cell captured by EM from each age confirmed that juxtavascular microglia contacted the basal lamina in vascular areas without full astrocyte endfoot coverage at all ages (Fig. 2.7 C, see also Movies 11 and 12). Similar to light microscopy (Fig. 2.1 H), the microglial surface area contacting the vessel decreased in older animals (Fig. 2.7 D; Unpaired student's t-test; n=29 cells (P5) and n=11 cells (P56); *p=.0155) and there were areas of the vasculature that were not fully ensheathed by astrocytes by EM, which were not contacted by microglia. Together, these data demonstrate that juxtavascular microglia contact

the vascular basal lamina and associate with the vasculature in areas lacking full coverage by astrocyte endfeet. The data raise the intriguing possibility that lack of astrocyte endfeet in early postnatal development provides a permissive environment for juxtavascular microglial association with and migration along the vasculature as they colonize the brain.

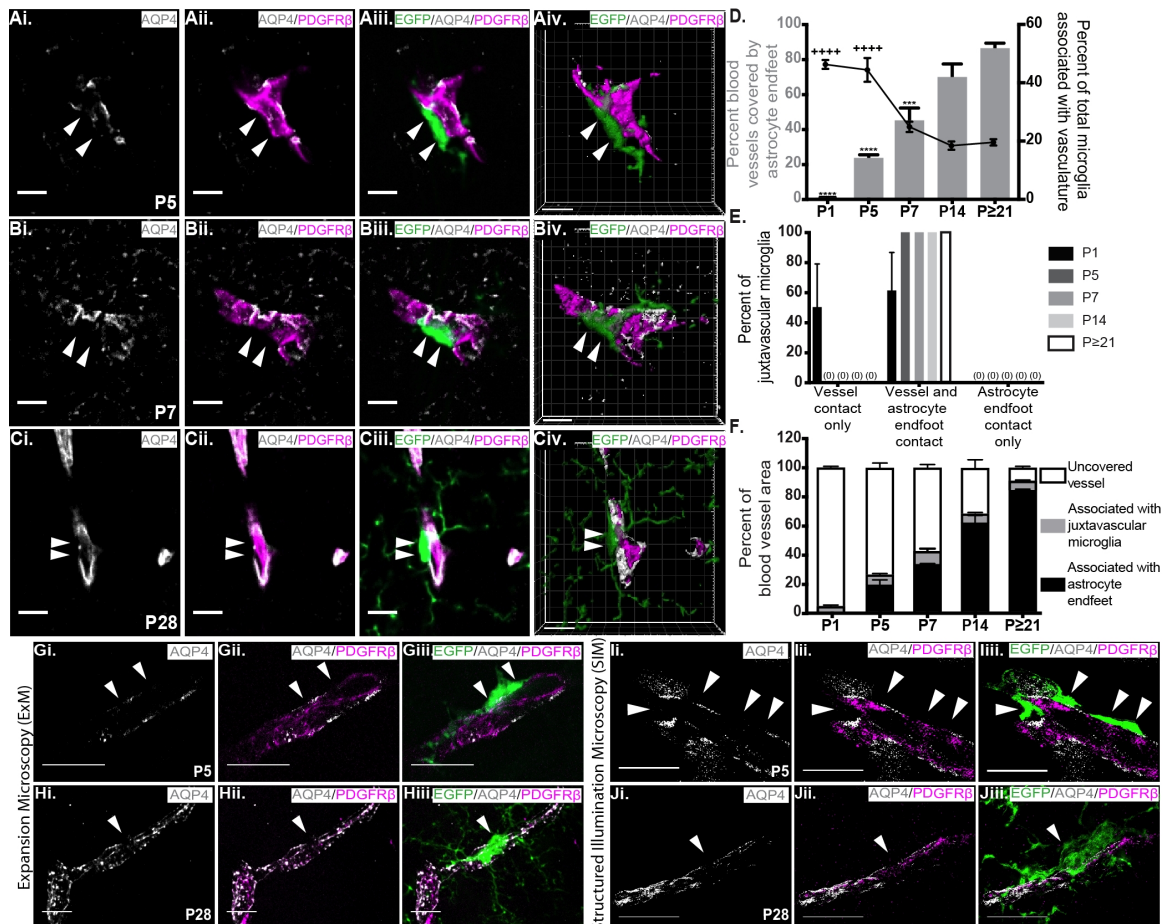
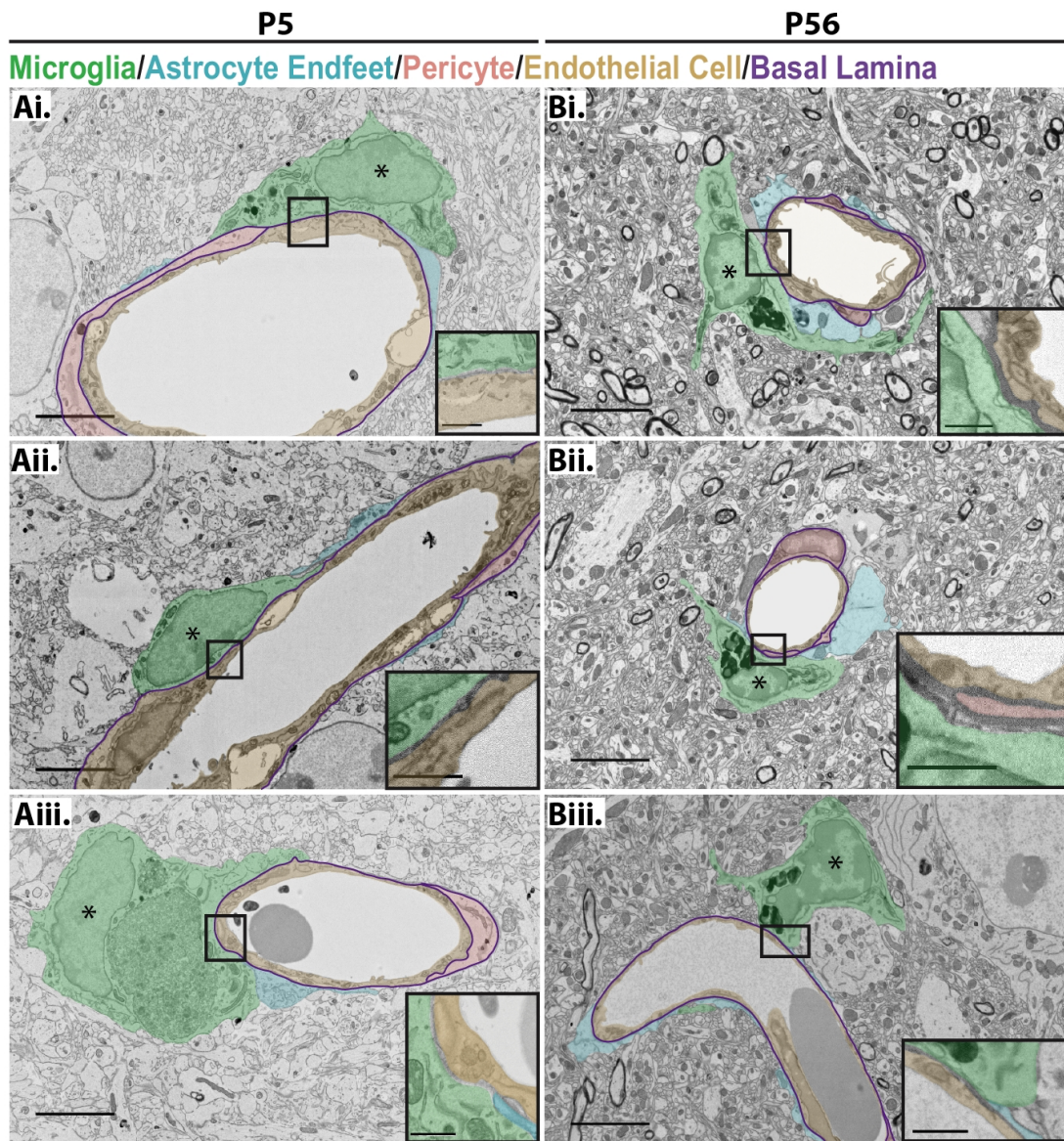


Figure 2.6: Juxtavascular microglia associate with the cortical vasculature in areas lacking full astrocytic endfoot coverage. **A-C.** Representative single optical plane images and 3D rendering (**Aiv-Civ**; see also Movies 8-10) of juxtavascular microglia (green, EGFP) and blood vessels (magenta, anti-PDGFR β) in areas void of astrocytic endfoot labeling (gray, anti-AQP4) in the frontal cortex at P5 (**A**), P7 (**B**) P28 (**C**). Filled arrowheads denote vascular areas that lack astrocyte endfeet where juxtavascular microglia are associated with the vessel. Scale bars= 10 μ m. **D.** Left Y axis, gray bars: quantification of the percent of blood vessels covered by astrocyte endfeet over development in the frontal cortex. One-way ANOVA with Dunnett's post hoc; comparison to P \geq 21, n=3 littermates per developmental time point, ***p=.0005, ****p<.0001. Right Y axis, black line: the percent of the total microglia population that are juxtavascular over development in the frontal cortex (data are the same as presented in Fig. 1G). One-way ANOVA with Dunnett's post hoc; comparison to P \geq 21, n=4 littermates per developmental time point, ++++p<.0001. **E.** Quantification of the percent of juxtavascular microglia associated with vessels only, vessels and astrocyte endfeet (representative images in A-C), and astrocyte endfeet only from 3D rendered images. **F.** Quantification of the percent of blood vessel area

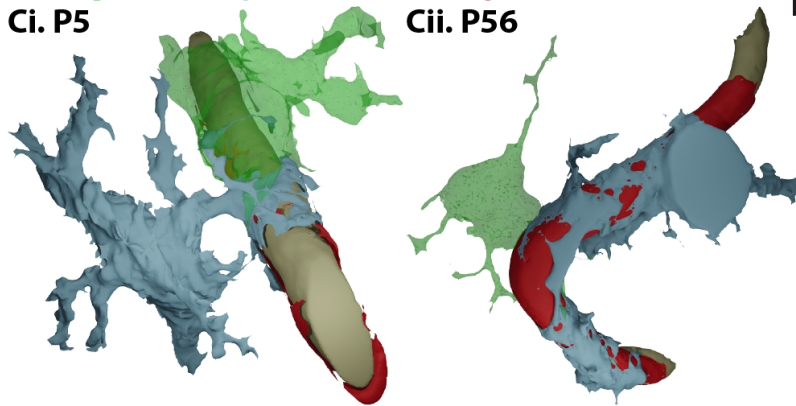
associated with astrocyte endfeet (black bars), juxtavascular microglia (gray bars), or uncovered vessels (white bars) in the frontal cortex over development. **G-J**. Representative expansion microscopy (ExM, **G-H**) and structured illumination microscopy (SIM, **I-J**) images of juxtavascular microglia (green, EGFP), in vascular areas lacking anti-AQP4 (gray) astrocytic endfoot labeling (filled arrowheads) in the P5 (**G, I**) and P28 (**H, J**) frontal cortex. Scale bars= 10 μ m. All error bars represent \pm SEM.



Microglia/Astrocyte Endfeet/Pericyte/Vessel Lumen

Ci. P5

Cii. P56



D.

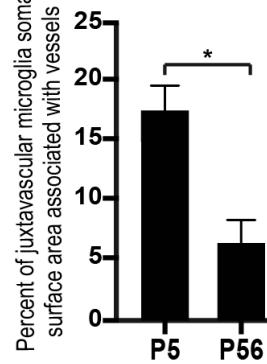


Figure 2.7: Ultrastructural analysis by EM reveals that juxtavascular microglia directly contact the basal lamina of the vasculature. A-B. Electron microscopy (EM) of juxtavascular microglia (green pseudocoloring) contacting the basal lamina (purple line) of a blood vessel in an area void of astrocyte endfeet (blue pseudocoloring) in the P5 (**A**, left column) and P56 (**B**, right column) frontal cortex. Pink pseudocoloring denotes a pericyte. Asterisks denote microglia nuclei. Scale bar= 5 μ m. The black box denotes the magnified inset in the bottom right corner where microglia (green pseudocoloring) directly contact the basal lamina (unlabeled in the inset) and only partially contacts the astrocyte endfoot (blue pseudocoloring). Scale bar= 1 μ m. **C.** 3D reconstruction of serial EM of P5 juxtavascular microglia (green pseudocoloring) in **Aiii (Ci)** and P56 juxtavascular microglia in **Biii (Cii)** contacting a blood vessel in an area void of astrocyte endfeet (blue pseudocoloring) (see also Movies 11 and 12). Red and tan pseudocoloring denotes a pericyte and vessel lumen, respectively. **D.** Quantification of the percent of juxtavascular microglia contacting the basal lamina in single optical plane images. Unpaired student's t-test; n=29 cells (P5) and n=11 cells (P56); *p=.0155. All error bars represent \pm SEM.

DISCUSSION

This study provides the first extensive analysis of juxtavascular microglia in the healthy developing and adult brain. We discovered that a high percentage of juxtavascular microglia are associated with largely capillaries in the early postnatal mouse cortex. Similar microglia-vascular association was observed in the developing human brain. Live imaging revealed that juxtavascular microglia are migratory along the vasculature during the peak of microglial colonization of the postnatal cortex and become stationary by adulthood. In addition, microglia are highly associated with the vasculature during development as they are being recruited to synapse-dense rich cortical regions and the timing of these interactions is regulated by CX3CR1. Last, we provide evidence that microglia preferentially associate and contact the vasculature at all ages in areas lacking full astrocyte endfoot coverage and expansion of astrocytic endfeet along blood vessels coincides with a decrease in microglia migration along vessels. Taken together, these data suggest that microglia are using the vasculature to migrate and colonize the cortex and the timing of this vascular association is regulated by CX3CR1. Our data further support a mechanism in which microglial migration along the vasculature during development ceases and juxtavascular microglia become stationary upon the maturation of astrocyte endfeet.

A possible role for the vasculature in regulating microglial colonization

Microglia are born as primitive macrophages in the embryonic yolk sac and enter the neuroepithelium at embryonic day E9.5 by crossing the pial surface and lateral ventricles (Navascués et al. 2000; Swinnen et al. 2013; Ginhoux et al. 2010). Microglia then migrate and proliferate through the brain parenchyma in a rostral-to-caudal gradient to colonize the embryonic brain (Sorokin et al. 1992; Navascués et al. 2000; Swinnen et al. 2013; Alliot, Godin, and Pessac 1999; Perry, Hume, and Gordon 1985; Ashwell 1991). Signaling mechanisms have been identified to regulate initial microglial infiltration into the brain parenchyma, such as matrix metalloproteinases (MMPs), stromal cell derived factor 1 (SDF-1), and Cxcl12/Cxcr4 signaling (Ginhoux et al. 2010; Arno et al. 2014; Ueno and Yamashita 2014). However, far less is known about the mechanisms regulating microglial localization to the appropriate brain regions once they reach the parenchyma, particularly during postnatal development. Previous work has shown microglia can migrate along the vasculature in acute embryonic brain slices and brain slices prepared from postnatal mice in an injury context (Smolders et al. 2017; Grossmann et al. 2002). In addition, other work has shown that oligodendrocyte precursor cells (OPCs) require the vasculature as a physical substrate for migration (Tsai et al. 2016). Similar findings have been identified for neural stem cells where the timing of astrocyte endfeet to the vessels has also been implicated (Bovetti et al. 2007; Fujioka, Kaneko, and Sawamoto 2019; Whitman et al. 2009). We have identified that microglia are highly associated with vasculature during the peak of microglial colonization and recruitment to

synapses. Furthermore, these vascular-associated microglia are migratory along blood vessels during early postnatal development and later become stationary once microglial colonization is complete. We also show in CX3CR1-deficient mice with known delays in microglial colonization of synapse-dense cortical regions that there are concomitant delays in microglial association with the vasculature. As we have observed no significant expression of *Cx3cl1* (the CX3CR1 ligand) by vascular cells (Gunner et al. 2019) and a subset of microglia still associate with the vasculature in *Cx3cr1^{-/-}* mice, this delay in microglial vascular association in *Cx3cr1^{-/-}* mice is most likely due to disruptions in chemokine gradient signaling from neuronal sources of CX3CL1 versus a direct effect of vascular adhesion. This would suggest that microglia receive directional cues from surrounding cells, use the vasculature as a substrate to migrate towards those cues, and the timing of this migration along the vasculature is regulated by CX3CR1. As *Cx3cr1^{-/-}* mice have delays in synapse maturation and pruning and, long-term, have behavioral deficits consistent with an autism-like phenotype, it suggests that these microglia-vascular associations in development have long-term consequences (Paolicelli et al. 2011; Zhan et al. 2014; Hoshiko et al. 2012). The vascular cues regulating microglial adhesion and migration in the healthy CNS are yet to be identified, which will be key to determine the relative importance of microglia-vascular interactions for microglial colonization, brain development, and long-term CNS function.

Microglia-astrocyte interactions at the NVU interface

Another interesting direction is to determine the role of astrocyte endfeet in regulating microglia-vascular interactions. Astrocytes are born and begin wrapping their processes to form endfeet along blood vessels during the first postnatal week (Daneman et al. 2010). By adulthood, astrocyte endfeet ensheath 60-95% of the vasculature (Mathiisen et al. 2010; Korogod, Petersen, and Knott 2015). Here, we demonstrate that juxtavascular microglia in the postnatal cortex represent a large percentage of total microglia and are migratory along the vasculature. Juxtavascular microglia migration decreases as astrocyte endfeet develop and ensheath the vasculature. In addition, we showed that microglia associate with vessels at all ages in areas lacking full astrocytic endfoot coverage and EM revealed contact between juxtavascular microglia and the vascular basal lamina. These data raise the intriguing possibility that the basal lamina provides an adhesive substrate for microglial association and migration, which becomes restricted upon astrocyte endfoot arrival. Astrocyte endfeet may, therefore, exclude microglia from contacting the basal lamina and associating with the vasculature. Another possibility is that microglia in the postnatal brain repel astrocyte endfeet, but this repellent signal later decreases as the animal matures so that astrocyte endfeet can wrap the vessels. Analysis of astrocyte endfoot-juxtavascular microglia interactions along blood vessels will be important going forward.

Possible functions for juxtavascular microglia in the healthy CNS

Are juxtavascular microglia a unique subpopulation of microglial cells that perform distinct functions at the NVU? Evidence in the literature suggests microglia play important roles in regulating the vasculature, but it is unclear if these functions are specific to juxtavascular microglia. For example, in the embryonic brain, microglia are often localized to vascular junction points and depletion of all microglia is associated with a decrease in vascular complexity (Fantin et al. 2010). Similar findings have been identified in the developing retina (Rymo et al. 2011; Checchin et al. 2006; Dudiki et al. 2020). Our data demonstrating that microglia are localized to the vasculature prior to the arrival of the astrocyte endfeet could place microglia in a position to regulate fine-scale remodeling of the vasculature throughout the brain and/or help to maintain the BBB prior to astrocyte endfoot arrival. Arguing against the latter, microglia depletion during development does not appear to induce changes in BBB integrity in the postnatal brain (Parkhurst et al. 2013; Elmore et al. 2014). These data are in contrast to the inflamed adult CNS, where microglia regulate BBB integrity (Zhao et al. 2018; Stankovic, Teodorczyk, and Ploen 2016). One of the most recent studies shows that during systemic inflammation, parenchymal microglia migrate to the vasculature and help to maintain the BBB at acute stages (Haruwaka et al. 2019). However, with sustained inflammation, microglia phagocytose astrocyte endfeet and facilitate BBB breakdown. In the absence of inflammation, it remains unknown what functions juxtavascular microglia may

perform. In the adult, this could be a simple consequence of tiling with no specific functional implications. Arguing against this, we found that juxtavascular microglia are less evenly distributed and closer to their nearest neighbor microglia compared to vascular-unassociated microglia within the cortex. Also, our *in vivo* live imaging data demonstrating microglia in the adult brain are stationary for nearly 2 months opens up the possibility that these cells could reside in a vascular niche. Although speculative at this point, one possible role could be to serve as immune surveillant “first responders”. It will be important going forward to determine whether these juxtavascular microglia in the adult have unique functions.

Microglia-vascular interactions: Implications for CNS disease

Our findings have important implications for neurological diseases associated with the injured or aged CNS where there is enhanced microglial association with the vasculature, such as in stroke, brain tumors, multiple sclerosis (MS), and Alzheimer’s disease (AD) (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018). This enhanced association can lead to further breakdown of the BBB and infiltration of peripheral immune cells into the CNS and possibly angiogenesis in the case of brain tumors (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018; Haruwaka et al. 2019). Therefore, understanding precisely when and where microglia interact with the vasculature in the healthy brain may lead to therapeutic strategies to reduce vascular

pathology and facilitate recovery. One intriguing possibility is that these sites of juxtavascular microglia association with vessels, which lack astrocyte endfeet, are more vulnerable to BBB breakdown and infiltration of peripheral immune cells and factors. In addition to neurodegenerative disorders, our findings may also have important implications for neurodevelopmental disorders such as autism spectrum disorders (ASDs). For example, microglia-vascular interactions may be important for the timing of microglial colonization to synapse-dense brain regions where they regulate synapse maturation and pruning during critical windows in development (Paolicelli et al. 2011; Hoshiko et al. 2012; Tremblay, Lowery, and Majewska 2010; Schafer et al. 2012; Gunner et al. 2019). If these interactions are disrupted, the timing of synapse development and, ultimately, neural circuit function may be altered. This is supported by our data from *Cx3cr1*^{-/-} mice showing delays in microglial association with the vessels, which is concomitant with known delays in microglial recruitment to developing synapses and delays in synapse maturation in these mice (Paolicelli et al. 2011; Zhan et al. 2014; Hoshiko et al. 2012). Long term, *Cx3cr1*^{-/-} mice have phenotypes associated with ASD, including decreased functional brain connectivity, deficits in social interactions, and increased repetitive behaviors (Zhan et al. 2014). However, a better understanding of how vascular interactions affect microglial colonization and extending these analyses of microglia-vascular interactions into the ASD human brain will be necessary.

Together, our work sheds new light on an understudied population of microglia, juxtavascular microglia. This work lays the foundation for identifying new molecular mechanisms underlying microglia-vascular interactions, identifying mechanistic underpinnings of microglia-astrocyte crosstalk at the level of the NVU, and furthering our understanding of juxtavascular microglia function in CNS homeostasis. With the vascular interface emerging as an important aspect of many neurological conditions, this study also lays the critical groundwork to study how this microglial population may be important in a wide range of CNS diseases.

MATERIALS AND METHODS

Animals

Male and female mice were used for all experiments. *Cx3cr1*^{-/-} mice (*Cx3cr1*^{EGFP/EGFP}; stock #005582) and *C57Bl6/J* (stock #000664) mice were obtained from Jackson Laboratories (Bar Harbor, ME). Heterozygous breeder pairs were set up for all experiments and wild-type (WT) and heterozygote littermates were used as controls with equal representation of males and females for each genotype. All experiments were performed in accordance with animal care and use committees and under NIH guidelines for proper animal welfare.

Human prenatal brain collection and immunofluorescence microscopy

Deidentified prenatal human brain tissues were collected via the Department of Pathology Autopsy Service at the University of California San Francisco under the approval of the Committee on Human Research (CHR, Study #: 12-08643). Brain tissues from four prenatal cases at 15, 18, 21 and 28 gestational weeks (GW) were evaluated using standard neuropathologic examinations to rule out any gross or microscopic abnormalities. These autopsy cases, which all had postmortem intervals of less than 48 hours, were fixed in freshly prepared 4% paraformaldehyde (PFA) and sampled at the level of the mammillary body. Following fixation in 4% PFA for 48 hours, brain samples were incubated with 20% sucrose solution, and were frozen in embedding medium OCT for cryosectioning at 20µm. For consistency, 3-6 consecutive sections were

prepared from each sample and immunostained with anti-Iba1 antibody (Wako; Richmond, VA; 1:3000) and anti-CD31 antibody (R&D Systems; Minneapolis, MN; 1:200). Images of the ventricular and subventricular zones at the level of the frontal cortex were acquired on Leica SP8 confocal microscope using a 40X (1.3NA) objective lens.

Preparation of tissue for immunofluorescence microscopy

Mice were perfused with 1X Hank's balanced salt solution (HBSS) -magnesium, -calcium, (Gibco, Gaithersburg, MD) prior to brain removal at indicated ages. For analysis of frontal and somatosensory cortex, brains were post-fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) for four hours. Brains were placed in 30% sucrose in 0.1M PB and allowed to sink prior to sectioning. Sections were blocked in 10% goat serum, 0.01% TritonX-100 in 0.1M PB for 1 hour before primary immunostaining antibodies were applied overnight. Secondary antibodies were applied for two hours the following day. All steps were carried out at room temperature with agitation. For structured illumination microscopy (SIM), sections were blocked in 3% BSA, 0.01% TritonX-100 in 0.1M PB for 1 hour before primary immunostaining antibodies were applied for 48 hours at 4°C. Secondary antibodies were applied for four hours at room temperature with agitation. The following antibodies were used: anti-P2RY12 (Butovsky Laboratory, Brigham and Women's Hospital, Harvard University; 1:200), anti-PECAM (Biolegend; San Diego, CA; 1:100), anti-aquaporin 4

(Millipore Sigma; St. Louis, Missouri; 1:200), anti-Pdgfr β (Thermo Fisher Scientific; Waltham, MA; 1:200), anti-Lyve1 (Abcam; Cambridge, MA; 1:200), anti-smooth muscle actin (SMA) (Millipore Sigma; St. Louis, Missouri; 1:200), and anti-VGluT2 (Millipore Sigma; St. Louis, Missouri; 1:2000).

Confocal microscopy

Immunostained sections were imaged on a Zeiss Observer Spinning Disk Confocal microscope equipped with diode lasers (405nm, 488nm, 594nm, 647nm) and Zen acquisition software (Zeiss; Oberkochen, Germany). For microglia-vascular interaction, microglial density, nearest neighbor analysis, microglia association with SMA+ or SMA- vessels and vascular density analyses, 20x, single optical plane, tiled images of the frontal or somatosensory cortex were acquired for each animal. To create a field of view (FOV), each tiled image was stitched using Zen acquisition software. Two FOVs (ie. tiled images) were acquired per animal. To note, anti-P2RY12 immunostaining was used to label microglia in wild type animals, which was more difficult to visualize at lower magnification at older ages compared to EGFP-labeled microglia. As a result, for anti-P2RY12 immunostained sections from P7-P28 animals, twelve 40x fields of view were acquired per animal with 76 z-stack steps at 0.22 μ m spacing. For analysis of juxtavascular microglia that occur by chance, vascular diameter, juxtavascular association with branched/unsegmented vessels, primary processes aligned with vessels, astrocyte endfeet/juxtavascular microglia

coverage on the vasculature, and vascular-associated microglia contacts with astrocytes, six-twelve 40X fields of view were acquired from the frontal cortex per animal with 76 z-stacks at 0.22 μ m spacing.

Juxtavascular microglia and microglia density analyses in the frontal and somatosensory cortices

Using the DAPI channel as a guide, a region of interest (ROI) was chosen in each cortical layer, I-VI from each 20x stitched tiled image (10 ROIs per animal). Subsequent images were analyzed in ImageJ (NIH; Bethesda, MD). For anti-P2RY12, sections were acquired at 40x, a maximum intensity projection was made from each z-stack and was considered a ROI (12 per animal). The ROI areas were recorded. The same ROI was transposed on the microglial channel and the cell counter ImageJ plugin was used to count the number of microglia in the ROI. The total density of microglia was then calculated by dividing the microglia number by the ROI area. To assess microglial association with the vasculature, the microglia and blood vessel channels were merged and the cell counter plugin was used to manually count the number of microglia with cell bodies directly apposed to blood vessels. Juxtavascular microglia were defined as microglia with at least 30% of their soma perimeter associated with blood vessels and soma centers that were within 10 μ m of the vessel. The percent of juxtavascular microglia was calculated by summing the total number of microglia associated with vasculature divided by the total number of microglia within the

ROI. To quantify the percent juxtavascular microglia that occur by chance six-twelve 40X fields of view were analyzed. The number of associations between microglia and blood vessels was quantified, then the orientation of the blood vessel channel was horizontally flipped 180 degrees and the number of associations between microglia and vasculature was re-quantified. For each animal, data from the ROIs were averaged together to get a single average per animal for statistical analyses.

Juxtavascular microglia analysis within the barrel cortex

Juxtavascular microglia analysis in the barrel cortex was performed blinded to genotype. Images were analyzed in ImageJ (NIH; Bethesda, MD). From each tiled image from each animal, 12-18 images containing VGluT2+ barrels were cropped for subsequent analyses. From each cropped image, the individual channels were separated and, using the free hand selection tool, each individual barrel was outlined. This ROI outlining the barrel was transposed to the microglia channel where the cell counter plugin was used to count the number of microglia in the barrels. The microglia and blood vessel channels were then merged and the same ROI was transposed onto the merged image. The cell counter plugin was used to count the number of microglia in barrels associated with vasculature. Each individual barrel ROI was then cleared, leaving behind only the septa fluorescence and the cell counter plugin was again used to count the number of microglia and the number of juxtavascular microglia in the septa. To calculate the

percent of juxtavascular microglia in the barrel cortex, the total numbers of juxtavascular microglia in the barrels and septa were summed and divided by the total number of microglia in the barrel and septa, respectively, for each ROI. The total microglia in barrels and septa, regardless of vascular association, were also calculated. All numbers across 12-18 cropped images were then averaged for a given animal prior to statistical analyses.

Vascular density analysis

Density analysis was performed blinded to genotype from the same tiled and stitched 20x images used for microglia-vascular association analyses. Using ImageJ (NIH; Bethesda, MD) software, the blood vessel channel was thresholded manually and the total blood vessel area was measured. Vascular density was calculated by dividing the blood vessel area by the area of the ROI. For each animal, the vascular density was averaged across all ROIs in the two FOV to get a single average per animal for statistical analyses.

Nearest neighbor analysis

Stitched 20x images, used for microglia-vascular association analyses, were used to determine nearest neighbor. Using ImageJ (NIH; Bethesda, MD) software, a ROI was drawn around the cortex. The microglia channel was thresholded so only somas were selected, the number of microglia was calculated using the analyze particles function, and microglia were manually

annotated as juxtavascular or vascular-unassociated microglia. The distance between nearest neighbor was calculated using the nearest neighbor distances plugin. For each animal, the nearest neighbor distance for juxtavascular and vascular-unassociated microglia was averaged across the two FOV to get a single average per animal for statistical analysis.

Microglial association with SMA+ or SMA- vessels analysis

Using the DAPI channel as a guide, a ROI was chosen in each cortical layer, I-VI from each 20x stitched tiled image (10 ROIs per animal). Subsequent images were analyzed in ImageJ (NIH; Bethesda, MD). The same ROI was transposed on the microglial, Pdgfr β , and SMA channel and the cell counter ImageJ plugin was used to count the total number of microglia, the number of juxtavascular microglia, and the number of juxtavascular microglia associated with SMA+ or SMA- vessels in the ROI. The percent of juxtavascular microglia associated with SMA+ or SMA- vessels was quantified by dividing the number of microglia on SMA+ or SMA- vessels by the number of total juxtavascular microglia. For each animal, data from the ROIs were averaged together to get a single average per animal for statistical analyses.

Vascular diameter analysis

Using Imaris (Bitplane) software, the diameter of the vessel was measured in 3D at microglial soma association points from 40X images (12 per animal). For each

animal, data from the 12 images were averaged together to get a single average per animal for statistical analysis.

Primary Process and branched/unsegmented vessel analyses

Using ImageJ (NIH; Bethesda, MD), the total number of primary processes, the number of primary processes aligned parallel with vessels, and whether the juxtavascular microglia was associated with a vessel branch point was calculated from 40X images (6 per animal, n=3-4 animals). The percent of primary processes aligned with vessels was calculated by dividing the number of primary processes aligned parallel and associated with vessels by the total number of primary processes. The percent of juxtavascular microglia associated with branched/unsegmented vessel was calculated by dividing the number of juxtavascular microglia associated with branched or unsegment vessels by the total number of juxtavascular microglia. For each animal, data from 6 images were averaged together to get a single average per animal for statistical analysis.

Acute Slice Time-Lapse Imaging

Mice were given a retro-orbital injection of Texas Red labeled dextran (Fisher Scientific; Waltham, MA) 10 minutes prior to sacrifice to label vasculature. Mice were euthanized at P7 or P \geq 120, brains were isolated and sectioned coronally at a thickness of 300 μ m using a Leica VT1200 vibratome in oxygenated 37°C artificial cerebrospinal fluid (ACSF). Slices were mounted on a MatTak glass

bottom microwell dish and placed in a Zeiss Observer Spinning Disk Confocal microscope equipped with diode lasers (405nm, 488nm, 594nm, 647nm) and Zen acquisition software (Zeiss; Oberkochen, Germany). Image acquisition started after a minimum of 30 minutes of tissue equilibration at 37°C with 5% CO₂ and within 2 hours of decapitation. Oxygenated artificial cerebral spinal fluid ACSF was continuously perfused over the slices at a rate of 1.5-2µm/minute for the duration of equilibration and imaging. Per animal, one field of view was imaged every 5 minutes over 6 hours on an inverted Zeiss Observer Spinning Disk Confocal and a 20X objective. Z-stacks spanning 50-60µm, with serial optical sections of 1.5-2µm were recorded from a minimal depth of 30µm beneath the surface of the slice to avoid cells activated by slicing.

***In vivo* 2-Photon Time-Lapse Imaging**

Cranial window surgeries were performed as previously described within the visual cortex (2.5µm lateral and 2.0 µm posterior from bregma) (Goldey et al. 2014). One week after surgery, mice were head-fixed to a custom-built running wheel and trained to run while head restrained for increasing time intervals several days a week. Two weeks post surgery long-term 2-photon live imaging began. Mice were given a retro-orbital injection of Texas Red labeled dextran (Fisher Scientific; Waltham, MA) 10 minutes prior to imaging and were head restrained on a custom built running wheel, which was positioned directly under the microscope objective. Images were acquired with a 20X water immersion

objective (Zeiss, NA 1.0) on a Zeiss Laser Scanning 7 MP microscope equipped with a tunable coherent Chameleon Ultra II multiphoton laser and BiG detector. Three different regions of interest (ROIs) were taken at least 75 μ m below the surface of the brain, with z-stacks spanning 45-65 μ m with a step size of 2.5 μ m for each animal. On the first day of imaging, each ROI was imaged every 5 minutes over 2 hours. The same ROIs were then imaged once (single z-stack) on the following days post first imaging session: 1, 3, 7, 10, 14, 17, 21, 24, 28, 35, and 42 days. For each imaging day, the ROIs from day 0 of imaging were identified based on the vascular structure.

Migration tracking and analysis

Image processing and microglial soma motility/migration tracking were performed using ImageJ (NIH; Bethesda, MD). Time series were first corrected for 3D drift using the 3D drift correction plugin (Parslow, Cardona, and Bryson-richardson 2014) and migration was tracked using the TrackMate plugin (Tinevez et al. 2017). For each developmental time point, 10-12 juxtavascular and vascular-unassociated microglia were analyzed per animal (n=4 mice per developmental time point). Only cells remaining in the field of view for six hours were included in the analysis. The average soma motility (μ m/h) was calculated by measuring the displaced distance of the microglial soma between time=0 min and time=360 min and dividing by the duration of the imaging session. Juxtavascular distance migrated was calculated by measuring the displaced distance of the microglial

soma between time=0 min and time=360 min. Juxtavascular migration trajectory was calculated by measuring the angle between the blood vessel and juxtavascular microglia soma along the longest, continuous stretch of motility on the vessel. Percent of cells within each binned category (motility, distance travelled, and trajectory) was calculated by dividing the number microglia of within each category by the total number of microglia. For each animal, data from each analyzed cell were averaged together to get a single average per animal for statistical analysis.

In vivo tracking of juxtavascular microglia motility and long-term juxtavascular microglia were performed using ImageJ (NIH; Bethesda, MD). Time series were first corrected for 3D drift using the 3D drift correction plugin (Parslow, Cardona, and Bryson-richardson 2014) and migration was tracked using the TrackMate plugin (Tinevez et al. 2017). To calculate percent of microglia stationary over two hours, the number of stationary juxtavascular and vascular-unassociated microglia was divided by the total number of microglia. To calculate the percent of original juxtavascular microglia that remain on vessels over 42 days, the number of juxtavascular microglia on day 0 was calculated. For each subsequent day, the number of these original juxtavascular microglia that were still associated with the vasculature was determined and divided by the number of original juxtavascular microglia on day 0. For each animal, data was analyzed from three ROIs and averaged together to get a single average per animal for statistical analysis.

Astrocyte endfeet and juxtavascular microglia coverage analysis

Using Imaris (Bitplane) software, the astrocyte endfeet, microglia, and vessel channels were 3D rendered from 40X images (6 per animal). The astrocyte and microglia channels were then masked onto the vessel channel and the masked astrocyte channel and microglia was 3D rendered. Volumes of the 3D rendered vessel channel, masked astrocyte endfeet channel, and masked microglia channel were recorded. The percent of blood vessels covered by astrocyte endfeet was calculated by dividing the blood vessel volume by the masked astrocyte endfeet volume. The percent of blood vessels covered by juxtavascular microglia was calculated by dividing the blood vessel volume by the masked microglia volume. Uncovered vessel volume was calculated by adding the masked astrocyte endfeet and juxtavascular microglia volume and subtracting from the vessel volume. The percent of juxtavascular microglia associated with vessels was calculated by dividing the microglial volume by masked microglial volume. For each animal, data from the 6 images were averaged together to get a single average per animal for statistical analysis.

Juxtavascular microglia- astrocyte association

Analysis was done using the same images used for astrocyte endfeet coverage analysis in Imaris (bitplane). The microglia was 3D rendered, masked onto the blood vessel and astrocyte endfeet channel, and the volume of the masked

microglial channel was recorded. The percent of juxtavascular microglia associated with blood vessels only, vessels and astrocyte endfeet, or astrocyte endfeet only was calculated by summing the number of microglia associated with vessels only, vessels and astrocyte endfeet, or astrocyte endfeet only and dividing by the total number of juxtavascular microglia. For each animal, data from the 6 images were averaged together to get a single average per animal for statistical analysis.

Expansion Microscopy (ExM)

Expansion microscopy was performed as previously described (Asano et al. 2018) with slight modification. Briefly, 80 μ m floating sections were blocked in 0.5% bovine serum albumin (BSA) and 0.3% Triton-X100 (TX-100) for 1 hour at room temperature. Primary antibodies, anti-aquaporin 4 (Millipore Sigma; St. Louis, Missouri; 1:200), anti-PDGFR β (Thermo Fisher Scientific; Waltham, MA; 1:100), and anti-GFP (Abcam; Cambridge, MA; 1:200) were incubated in 0.5% BSA and 0.3% TX-100 at 4°C for 4 nights. Secondary antibodies were added at 1:200 dilutions overnight at room temperature. Expansion microscopy protocol (Basic Protocol 2) was then followed as published in Asano et al. 2018.

Structured Illumination Microscopy (SIM)

Structured Illumination Microscopy (SIM) was performed using a GE Delta Vision OMX V4 microscope with pCO.edge sCMOS cameras and an Olympus 60x 1.42

NA objective. Samples were mounted in Prolong Glass mounting media with #1.5 coverslips and imaged using 1.516 refractive index immersion oil. Image processing was completed using the GE softWorx software and image quality was determined using the SIMcheck plugin in ImageJ. SIM figures were produced in ImageJ (NIH; Bethesda, MD).

Scanning Electron Microscopy (SEM)

Mice were perfusion fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer at pH 7.4 (Science Services). Brains were dissected, vibratome sectioned, and immersion fixed for 24h at 4°C. We applied a rOTO (reduced osmium-thiocarbohydrazide-somium) staining procedure adopted from Tapia et al. (Tapia et al. 2013). Briefly, the tissue was washed and post-fixed in 2% osmium tetroxide (EMS), 2% potassium hexacyanoferrate (Sigma) in 0.1 M sodium cacodylate buffer. After washes in buffer and water the staining was enhanced by reaction with 1% thiocarbohydrazide (Sigma) for 45 min at 50°C. The tissue was washed in water and incubated in 2% aqueous osmium tetroxide. All osmium incubation steps were carried out over 90 min with substitution by fresh reagents after 45 min, respectively. To further intensify the staining, 2% aqueous uranyl acetate was applied overnight at 4°C and subsequently warmed to 50°C for 2h. The samples were dehydrated in an ascending ethanol series and infiltrated with LX112 (LADD). The samples were flat embedded into gelatin capsules (Science Services) and cured for 48h. The

block was trimmed by 200 μm at a 90° angle on each side using a TRIM90 diamond knife (Diatome) on an ATUMtome (Powertome, RMC). Consecutive sections were taken using a 35° ultra-diamond knife (Diatome) at a nominal cutting thickness of 100 nm and collected on freshly plasma-treated (custom-built, based on Pelco easiGlow, adopted from Mark Terasaki) CNT tape (Yoshiyuki Kubota et al. 2018). We collected 450 (P5) and 550 (P56) cortical sections, covering a thickness of 45-55 μm in depth. Tape strips were mounted with adhesive carbon tape (Science Services) onto 4-inch silicon wafers (Siegert Wafer) and grounded by additional adhesive carbon tape strips (Science Services). EM micrographs were acquired on a Crossbeam Gemini 340 SEM (Zeiss) with a four-quadrant backscatter detector at 8 kV. In ATLAS5 Array Tomography (Fibics), the whole wafer area was scanned at 3000 nm/pixel to generate an overview map. The entire ultrathin section areas of one wafer (314 sections (P5), 279 sections (P56) were scanned at 100 x 100 x 100 nm³ (465 x 638 μm^2 (P5), 1249 x 707 μm^2 (P56). After alignment in Fiji TrakEM2 (A. Cardona et al. 2012) areas that contained microglia in close proximity to blood vessels (148 x 136 x 16 μm^3 (P5), 193 x 186 x 12 μm^3 (P56) were selected for high resolution acquisition. We collected 29 total 2D micrographs (10 x 10 nm²) from n=3 animals at P5 and 11 total micrographs from n=3 animals at P56. From each age, one juxtavascular microglia was identified and selected to generate a 3D volume (10 x 10 x 100 nm³). The image series were aligned in TrakEM2 using a series of automated and manual processing steps. For the P5 and P56 image

series, segmentation and rendering was performed in VAST (Volume And Segmentation Tool) (Berger et al. 2018). We used Blender to render the two 3D models (Community 2018). The percent of juxtavascular microglia contacting basal lamina was calculated using ImageJ (NIH; Bethesda, MD). Single optical plane image were opened, the perimeter area of microglia was measured, and the length of contact between microglia and the basal lamina was measured. The percent juxtavascular microglia contacting the basal lamina was calculated by dividing the perimeter of the microglia by the length of contact between microglia and basal lamina.

Experimental Design and Statistical analyses

GraphPad Prism 7 (La Jolla, CA) provided the platform for all statistical and graphical analyses. The ESD method was run for each ROI per animal to identify outliers. Significant outliers were removed prior to analyses. Analyses included Students t-test when comparing 2 conditions or one-way ANOVA followed by Dunnett's post hoc analysis or two-way ANOVA followed by Sidak's or Tukey's post hoc analyses (indicated in results figure legends).

MOVIE LEGENDS

Movie 1: 3D rendering of juxtavascular microglia in the early postnatal frontal cortex. 3D reconstruction and surface rendering of juxtavascular microglia (green, EGFP) associated with blood vessels (magenta, anti-PECAM) in the P5 frontal cortex. Yellow denotes association area between microglia and blood vessels.

Movie 2: 3D rendering of juxtavascular microglia in the P28 frontal cortex. 3D reconstruction and surface rendering of juxtavascular microglia (green, EGFP) associated with blood vessels (magenta, anti-PECAM) in the P28 frontal cortex. Yellow denotes association area between microglia and blood vessels.

Movie 3: Juxtavascular microglial migration in the early postnatal somatosensory cortex. Representative live imaging of juxtavascular microglia (green, EGFP) migrating on vessels (magenta; dextran) in the P7 somatosensory. *Cx3cr1^{EGFP/+}* mice received a retro-orbital injection of Texas red labeled dextran to label the vasculature 10 minutes prior to euthanasia. Coronal somatosensory cortices were imaged every 5 minutes over 6 hours immediately following slice preparation.

Movie 4: Juxtavascular microglial migration in the early postnatal somatosensory cortex. A second representative live imaging of juxtavascular

microglia (green, EGFP) migrating on vessels (magenta; dextran) in the P7 somatosensory. *Cx3cr1^{EGFP/+}* mice received a retro-orbital injection of Texas red labeled dextran to label the vasculature 10 minutes prior to euthanasia. Coronal somatosensory cortices were imaged every 5 minutes over 6 hours immediately following slice preparation.

Movie 5: Juxtavascular microglial migration in the early postnatal somatosensory cortex. A third representative live imaging of juxtavascular microglia (green, EGFP) migrating on vessels (magenta; dextran) in the P7 somatosensory. *Cx3cr1^{EGFP/+}* mice received a retro-orbital injection of Texas red labeled dextran to label the vasculature 10 minutes prior to euthanasia. Coronal somatosensory cortices were imaged every 5 minutes over 6 hours immediately following slice preparation.

Movie 6: Juxtavascular microglial migration in the adult somatosensory cortex. Representative live imaging of juxtavascular microglia (green, EGFP) stationary on vessels (magenta; dextran) in the P \geq 120 somatosensory cortex. *Cx3cr1^{EGFP/+}* mice received a retro-orbital injection of Texas red labeled dextran to label the vasculature 10 minutes prior to euthanasia. Coronal somatosensory cortices were imaged every 5 minutes over 6 hours immediately following slice preparation.

Movie 7: 2-photon *in vivo* live imaging of juxtavascular microglia in the adult cortex. Representative 2-photon *in vivo* live imaging of juxtavascular microglia (green, EGFP) stationary on blood vessels (magenta, dextran) over 2 hours *in vivo* in the adult cortex. *Cx3cr1*^{EGFP/+} mice received a retro-orbital injection of Texas Red-labeled dextran to visualize the vasculature 10 min prior to each imaging session. EGFP+ juxtavascular microglia were then imaged every 5 minutes for 2 hours.

Movie 8: Juxtavascular microglia associate with the cortical vasculature in areas lacking full astrocytic endfoot coverage in the P5 frontal cortex. 3D reconstruction and surface rendering of juxtavascular microglia (green, EGFP) associated with blood vessels (magenta, anti-PDGFR β) in areas void of astrocytic endfoot labeling (gray, anti-AQP4) in the frontal cortex at P5.

Movie 9: Juxtavascular microglia associate with the cortical vasculature in areas lacking full astrocytic endfoot coverage in the P7 frontal cortex. 3D reconstruction and surface rendering of juxtavascular microglia (green, EGFP) associated with blood vessels (magenta, anti-PDGFR β) in areas void of astrocytic endfoot labeling (gray, anti-AQP4) in the frontal cortex at P7.

Movie 10: Juxtavascular microglia associate with the cortical vasculature in areas lacking full astrocytic endfoot coverage in the P28 frontal cortex. 3D

reconstruction and surface rendering of juxtavascular microglia (green, EGFP) associated with blood vessels (magenta, anti-PDGFR β) in areas void of astrocytic endfoot labeling (gray, anti-AQP4) in the frontal cortex at P28.

Movie 11: Serial EM 3D reconstruction of juxtavascular microglia in the early postnatal cortex. 3D reconstruction of serial electron microscopy (EM) of juxtavascular microglia (green) contacting a blood vessel in an area void of astrocyte endfeet (blue) in the P5 frontal cortex. Red and tan pseudocoloring denotes a pericyte and vessel lumen, respectively.

Movie 12: Serial EM 3D reconstruction of juxtavascular microglia in the P56 cortex. 3D reconstruction of serial electron microscopy (EM) of juxtavascular microglia (green) contacting a blood vessel lacking full astrocyte endfoot (blue) coverage in the P56 frontal cortex. Red and tan pseudocoloring denotes a pericyte and vessel lumen, respectively.

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CHAPTER III

The fractalkine receptor regulates the timing of microglia-vasculature interactions independent of fractalkine

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Contribution Summary

E.M. collected all images, performed and analyzed adhesion assay, and quantified all results. S.C.B. performed *in situ* hybridization. S.A.L. provided mice. E.M. and D.P.S. designed and interpreted all experiments. E.M. wrote the final document.

ABSTRACT

Microglia, the resident central nervous system (CNS) macrophage, are born in the yolk sac and take up residence in the CNS during embryonic development. Once in the brain parenchyma, microglia become highly associated with the vasculature and migrate along vessels as they colonize developing synapse-dense brain regions. While the timing of microglia-vascular interactions and colonization of the barrel cortex is dependent on the fractalkine receptor (CX3CR1), the precise mechanism by which CX3CR1 regulates the timing of juxtavascular microglia, and subsequent colonization, is unknown. Here, we identify that CX3CR1 modulates the timing of early developmental microglial-vascular interactions across cortical brain regions. Further, we show that CX3CR1 facilitates microglial adhesion to endothelial cells in young postnatal mice, a time of active microglial colonization, but not older mice. Finally, we demonstrate that the timing of microglia-vascular interactions in the developing brain is independent of the canonical CX3CR1 ligand, fractalkine (CX3CL1). Together, these data provide mechanistic insight into the CX3CR1-dependent timing of microglia-vascular interactions. They further suggest a role for CX3CR1-dependent microglial adhesion to blood vessels, independent of CX3CL1, in regulating the timing of microglial colonization of synapse-dense brain regions.

INTRODUCTION

Microglia, the resident immune cells of the CNS, play an intricate role in an array of developmental processes, including regulating neurite outgrowth, synaptogenesis, and synaptic pruning (Schafer et al. 2012; Pont-lezica et al. 2014; Nagata et al. 1993; Miyamoto et al. 2016). To perform these critical processes, microglia must localize to the correct brain region at the appropriate time. In the developing brain, a high percent of the microglial population is juxtavascular and migrate on blood vessels as they colonize the brain (Mondo et al. 2020). The timing of microglia-vascular interactions and subsequent localization into synaptic dense brain regions within the somatosensory cortex is dependent on the fractalkine receptor (CX3CR1) (Hoshiko et al. 2012; Mondo et al. 2020), however precisely how CX3CR1 regulates the timing of juxtavascular microglia is unknown. Answers to this question will have an important impact on our understanding of how microglia reach the appropriate brain region at the correct time and could provide insight into disorders of the developing brain such as autism spectrum disorders (ASDs), where disruptions in microglia density and synaptic connectivity during critical periods in development, have been identified (Edmonson, Ziats, and Rennert 2016; Morgan et al. 2010).

CX3CR1 is a 7 transmembrane domain G-protein-coupled chemokine receptor that is highly enriched in microglia in the CNS (Combadiere, Ahuja, and Murphy 1995; Raport et al. 1995). Broadly, CX3CR1 regulates the timing of microglial localization in the developing brain. For example, CX3CR1 knockout

mice (*Cx3cr1*^{-/-}) have a delay in recruitment of microglia to synapse-dense regions of the hippocampus and somatosensory cortex, which is concomitant with a delay in synapse development (Paolicelli et al. 2011; Hoshiko et al. 2012; Zhan et al. 2014). One study assessed microglial colonization and subsequent effects on synapse maturation in *Cx3cr1*^{-/-} mice in a sub-region of the somatosensory cortex called the barrel cortex (Hoshiko et al. 2012). This study found that microglia within layer IV of the barrel cortex, are recruited to dense areas of thalamocortical synapses, termed barrel centers, at postnatal day (P) 7. This process is delayed in *Cx3cr1*^{-/-} mice, which is accompanied by a delay in the functional maturation of synapses, as measured by NMDA receptor subunit composition and AMPA/NMDA ratios (Hoshiko et al. 2012). Similar delays in microglial recruitment to synapses and subsequent delays in synapse maturation were also observed in the hippocampus of postnatal *Cx3cr1*^{-/-} mice (Paolicelli et al. 2011). Long term, *Cx3cr1*^{-/-} mice have deficits in functional connectivity, as well as ASDs-like behaviors including defects in social interactions and increased repetitive behaviors (Zhan et al. 2014).

Along with delays in localization to synapse-dense brain regions, *Cx3cr1*^{-/-} mice have delays in microglial localization to the vasculature. In the embryonic and early postnatal developing brain, microglia are highly juxtavascular, associating with and migrating along the vasculature as they actively colonize the cortex (Fantin et al. 2010; Smolders et al. 2017; Mondo et al. 2020). In the barrel cortex, microglia are associated with the vasculature as they colonize the

synapse-dense rich barrel centers and these interactions are delayed in *Cx3cr1*^{-/-} mice, concomitant with delays in microglial localization into barrel centers (Mondo et al. 2020). How CX3CR1 regulates the timing of juxtavascular microglia is an open question. In the periphery, CX3CR1 and its canonical and only known *in vivo* ligand, fractalkine (CX3CL1), have been implicated in regulating interactions between immune cells and the vasculature through cellular adhesion (Fong et al. 1998; Hamon et al. 2017; Schwarz et al. 2010; Imai et al. 1997; Imaizumi, Yoshida, and Satoh 2004; Muehlhoefer et al. 2000; Umehara et al. 2004; Johnson and Jackson 2013; Goda et al. 2000). Whether CX3CR1 regulates the timing of microglia-vascular interactions through cellular adhesion and if CX3CL1 signaling is involved in the developing brain is unknown.

In the current study, we identify that CX3CR1 regulates the timing of microglia-vascular interactions throughout the developing rodent cortex. We demonstrate that CX3CR1 deficient microglia isolated from early postnatal mice are less adhesive to endothelial cells *in vitro* compared to older mice. Finally, we show that the timing of microglia-vascular interactions in the early postnatal brain is independent of CX3CL1, which is not highly expressed in vascular endothelial cells in the brain during early postnatal development. Together, these data provide mechanistic insight into the CX3CR1-dependent microglia-vascular interactions. They further suggest a role for CX3CR1 in regulating the timing of microglial recruitment to synapses via cell adhesion with blood vessels, independent of CX3CL1.

RESULTS

CX3CR1 modulates the timing of early developmental juxtavascular microglia across cortical brain regions.

During rodent and human development, microglia somas are in close association with blood vessels and the timing of these interactions is dependent on CX3CR1 signaling in the rodent barrel cortex (Fantin et al. 2010; Monier et al. 2007; Checchin et al. 2006; Mondo et al. 2020). We set out to explore whether the timing of microglia-vascular interactions were more globally impaired in CX3CR1 deficient (*Cx3cr1*^{-/-}) mice across development. We focused our analysis on the frontal cortex where microglia are highly juxtavascular between postnatal day (P)1-P5 (Mondo et al. 2020). Microglia were labeled using transgenic mice that express EGFP under the control of the fractalkine receptor CX3CR1, *Cx3cr1*^{EGFP/+} (heterozygous mice, *Cx3cr1*^{+/-}) or *Cx3cr1*^{EGFP/EGFP} (homozygous knockout mice, *Cx3cr1*^{-/-}). Microglia-vascular interactions in *Cx3cr1*^{+/-} phenocopy wild-type (WT) mice (Mondo et al. 2020), therefore they are used as control mice here. The vasculature was labeled with an antibody against platelet endothelial cell adhesion molecule (PECAM). As previously described (Mondo et al. 2020), we observed a high percentage of juxtavascular microglia during early postnatal development in *Cx3cr1*^{+/-} mice (Fig 3.1 A,C). Similar to the barrel cortex, the timing of microglia-vascular interactions in the frontal cortex was dependent on CX3CR1 (Fig 3.1 B-C). We observed a significant reduction in the percent of the total microglial population associated with blood vessels in *Cx3cr1*^{-/-} mice

compared to controls during early postnatal development (Fig 3.1 A-C). We confirmed this disruption in the timing of microglia-vascular interactions in *Cx3cr1*^{-/-} mice was independent of changes in microglial and vascular density (Fig 3.1 D-E). Further, we confirmed this CX3CR1-dependent timing of microglia-vascular interactions in the frontal cortex in a second model of CX3CR1 deficiency (*Cx3cr1*^{CreER/CreER}) (Fig 3.1 F). Together, these data demonstrate that CX3CR1 is a more global regulator of microglia-vascular interactions within the developing cortex.

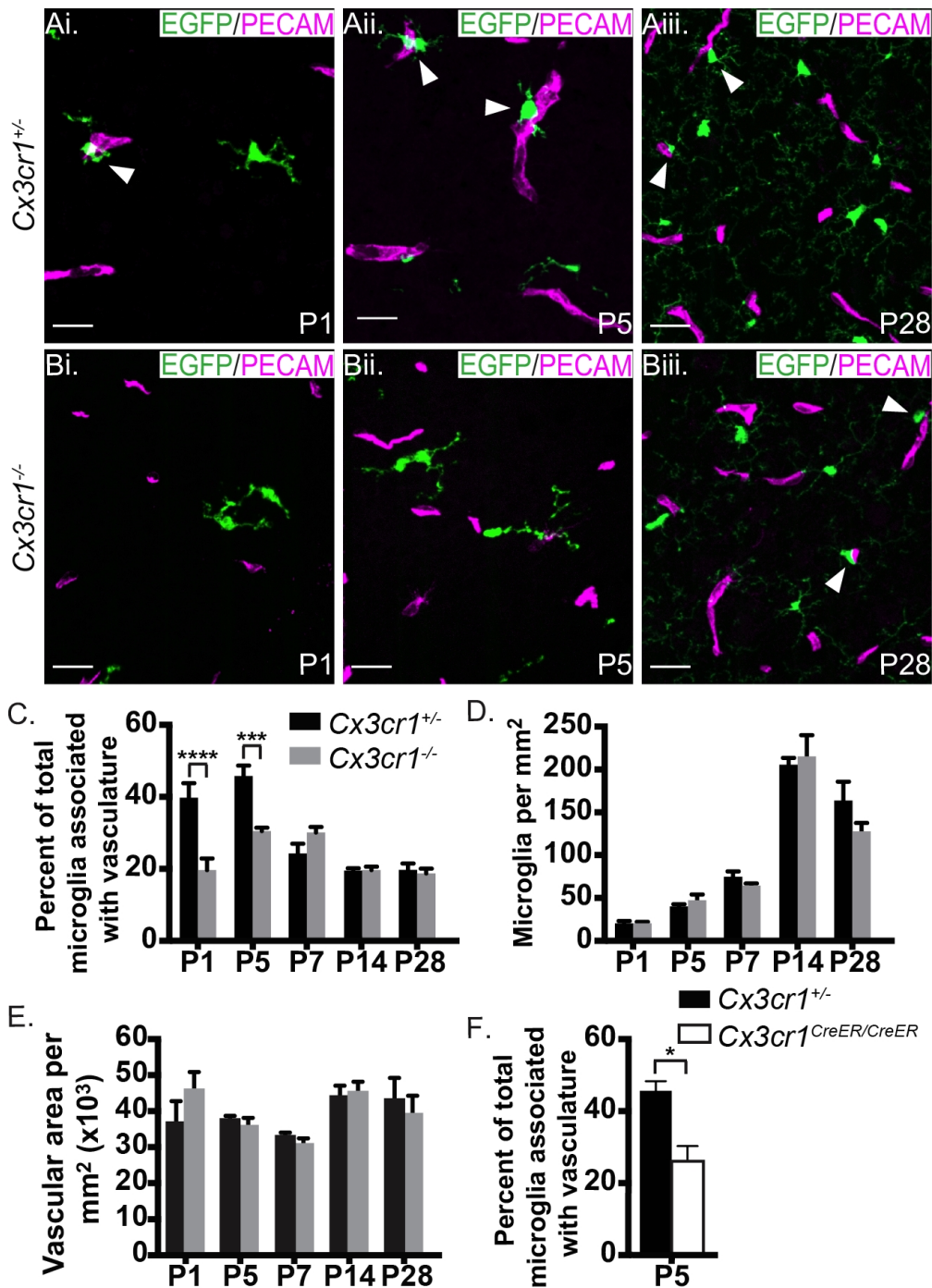


Figure 3.1: The timing of microglia-vascular associations in the early postnatal frontal cortex is dependent on CX3CR1. A-B. Representative images of microglia (green, EGFP) and vasculature (magenta, anti-PECAM) in $Cx3cr1^{+/-}$ (A) and $Cx3cr1^{-/-}$ (B) mice at P1 (Ai, Bi), P5 (Aii, Bii), and P28 (Aiii, Biii) in the frontal cortex. Microglia are labeled by transgenic expression of EGFP

and vasculature is immunolabeled by anti-PECAM. White arrowheads denote juxtavascular microglia. Scale bar= 20 μ m. **C.** Quantification of the percent of total microglia associated with vasculature in *Cx3cr1*^{+/-} (black bars) and *Cx3cr1*^{-/-} (gray bars) littermates over development in the frontal cortex shows a significant reduction in association at P1 and P5 in *Cx3cr1*^{-/-} animals. (Two-way ANOVA with a Sidak's post hoc; n=4 littermates per genotype per developmental time point, ***p<.001, ****p<.0001). **D.** Quantification of microglial density in *Cx3cr1*^{+/-} (black bars) and *Cx3cr1*^{-/-} (gray bars) over development in the frontal cortex demonstrates no significant difference in *Cx3cr1*^{-/-} mice. (Two-way ANOVA with a Sidak's post hoc; n=4 littermates per genotype per developmental time point). **E.** Quantification of vascular density in *Cx3cr1*^{+/-} (black bars) and *Cx3cr1*^{-/-} (gray bars) over development in the frontal cortex (One-way ANOVA with Dunnett's post hoc; comparison to P28, n=4 mice per genotype per developmental time point). **F.** Quantification of the percent of total microglia associated with vasculature in the frontal cortex of a second model of CX3CR1 deficiency, *Cx3cr1*^{CreER/CreER} (white bar) compared to *Cx3cr1*^{+/-} (black bar) controls (Unpaired student's t-test; n=4 mice per genotype, *p<.05).

CX3CR1 deficient microglia isolated from young, postnatal mice are less adhesive to endothelial cells

We next set out to understand the mechanism by which CX3CR1 regulates the timing of microglia-vascular interactions across the cortex during early postnatal development. In the periphery, CX3CR1 mediates immune cell-vasculature interactions through adhesion to its only known *in vivo* ligand, CX3CL1 (Fong et al. 1998; Hamon et al. 2017; Schwarz et al. 2010; Imai et al. 1997; Imaizumi, Yoshida, and Satoh 2004; Muehlhoefer et al. 2000; Umehara et al. 2004; Johnson and Jackson 2013; Goda et al. 2000). First, we explored whether CX3CR1 signaling is modulating microglia-vascular adhesion by performing an *in vitro* cell adhesion assay (Lowe and Raj 2015). In this assay, microglia are acutely isolated from P3-P5 or P14 mouse cortices and are then added to cultured, adherent endothelial cells (HUVEC) for 30-60 min. The non-adherent microglia are then washed away and the remaining microglia that have adhered to the HUVEC cells are quantified. Using this assay, we observed a significant reduction in the endothelial adherence of acutely isolated *Cx3cr1*^{-/-} microglia compared to *Cx3cr1*^{+/-} microglia from P3-P5 mice, but not from P14 mice (Fig. 3.2 A-F). These development differences in adhesion are concomitant with a reduction in the percent of microglia-vascular interactions we observed in *Cx3cr1*^{-/-} mice at P1-P5, but not at P14 (Fig. 3.1). These data establish that CX3CR1 deficient microglia are less adhesive to endothelial cells when isolated from early postnatal development and suggests that the delays observed in the

timing of juxtavascular microglia in *Cx3cr1^{-/-}* mice may be through CX3CR1-dependent vascular adhesion.

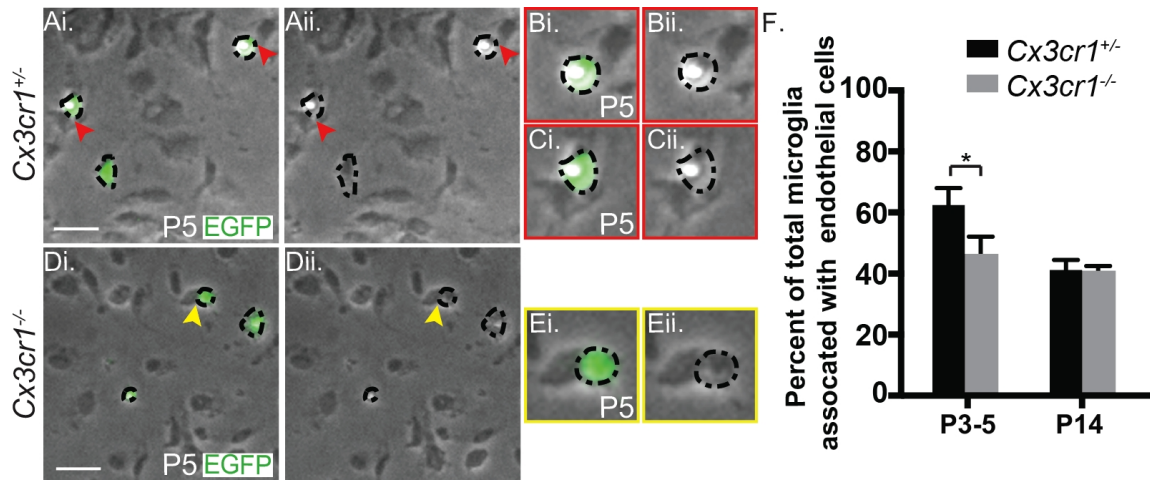


Figure 3.2: CX3CR1 deficient microglia isolated from early postnatal development are less adhesive to endothelial cells. A-E. Representative images of the cell adhesion assay, which includes EGFP fluorescence to visualize microglia isolated from *Cx3cr1*^{+/+} (A-C) and *Cx3cr1*^{-/-} (D-E) mice at P5, overlaid over phase contrast to visualize endothelial cells (HUVECs) (Ai-Ei) and phase contrast images only (Aii-Eii). Black dotted lines denote EGFP+ microglia. Red and yellow arrowheads indicate microglia contact points with endothelial cells, which are magnified in B-C (red, *Cx3cr1*^{+/+}) and E (yellow, *Cx3cr1*^{-/-}). Scale bar= 50 μ m. F. Quantification of the percent of total *Cx3cr1*^{+/+} (black bars) or *Cx3cr1*^{-/-} (gray bars) microglia acutely isolated from P3-5 or P14 mice that adhered to endothelial cells. (Two-way ANOVA with a Sidak's post hoc; n=4 biological replicates; n=1-2 technical replicates per biological replicate; *p<.05). All error bars represent \pm SEM.

The timing of microglia-vascular interactions is independent of the canonical CX3CR1 ligand fractalkine (CX3CL1).

To investigate if CX3CR1 mediates microglia-vascular adhesion through CX3CL1, we assessed the timing of these interactions in the frontal cortex of CX3CL1-deficient mice (*Cx3cl1*^{-/-}), in which microglia were similarly labeled by transgenic expression of EGFP in the wild-type and knockout littermates (*Cx3cr1*^{+/-}; *Cx3cl1*^{+/+} and *Cx3cr1*^{+/-}; *Cx3cl1*^{-/-}, respectively). CX3CL1 is the canonical and only known *in vivo* ligand of CX3CR1, which is highly enriched in neurons in the CNS and is expressed by vascular endothelial cells in the periphery (Bazan et al. 1997; Imai et al. 1997; Imaizumi, Yoshida, and Satoh 2004). Surprisingly, unlike *Cx3cr1*^{-/-} mice, microglia still associate with the vasculature in early postnatal *Cx3cl1*^{-/-} mice at levels comparable to littermate controls (Fig 3.3 A-B). Similar to *Cx3cr1*^{-/-} mice, there were no significant changes in microglial or vascular density when compared to *Cx3cl1*^{+/-} littermates (Fig 3.3 C-D).

We further explored *Cx3cl1* expression in endothelial cells by performing multiplex *in situ* hybridization for *Cx3cl1* and *Pecam* in *Cx3cr1*^{+/-} animals (Fig 3.4 A). We found *Cx3cl1* was not highly expressed within *Pecam* positive cells. In contrast, *Cx3cl1* was highly enriched in surrounding cells with nuclei >5 μm (Fig 3.4 A-B), which are likely neurons. This is consistent with previous work showing *Cx3cl1* enrichment in neurons and little expression in endothelial cells in the cerebral cortex (Sunnemark et al. 2005; Ye Zhang et al. 2014). These data

suggest that the timing of CX3CR1-dependent microglia-vascular interactions in the developing brain is independent of the only known *in vivo* CX3CR1 ligand, CX3CL1.

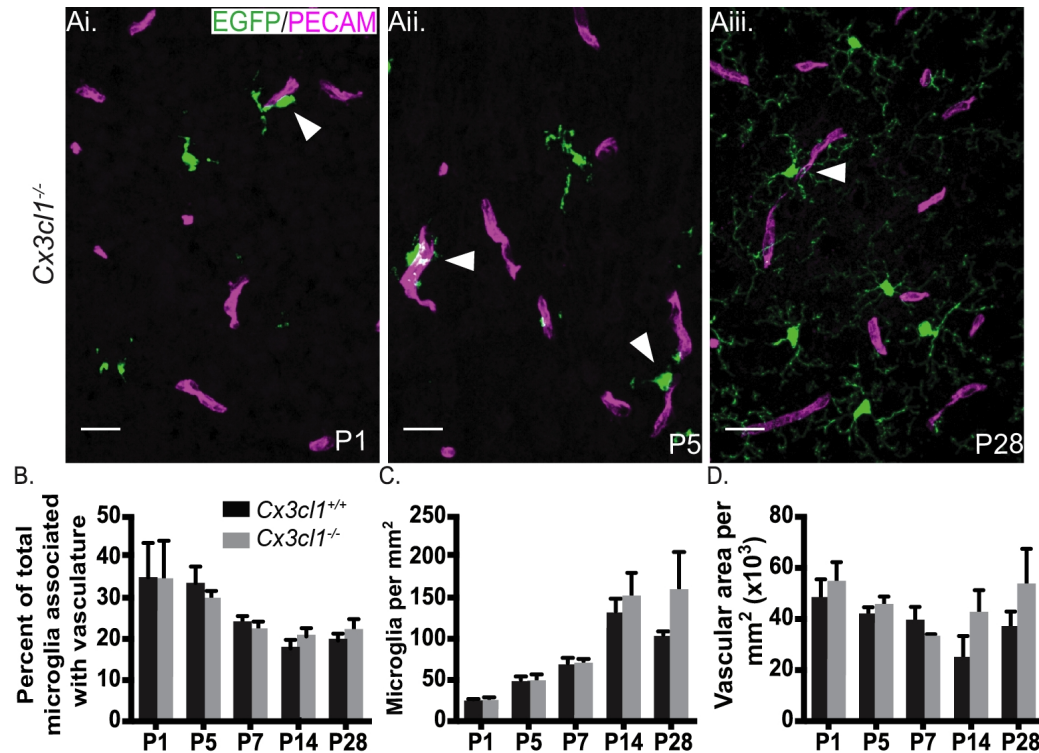


Figure 3.3: The timing of microglia-vascular associations in the early postnatal brain are independent of CX3CL1. **A.** Representative images of microglia (green, EGFP) and vasculature (magenta, anti-PECAM) from P1 (**Ai**), P5 (**Aii**), and P28 (**Aiii**) *Cx3cl1*^{-/-} mice frontal cortex. Note, for transgenic expression of microglia, the *Cx3cl1*^{EGFP/+} reporter mouse line was crossed into *Cx3cl1*^{-/-} mice and littermate controls. Therefore, all mice are *Cx3cl1*^{+/+}. White arrowheads denote juxtavascular microglia. Scale bar= 20 μ m. **B.** Quantification of the percent of microglia associated with vasculature in *Cx3cl1*^{+/+} (black bar) and *Cx3cl1*^{-/-} (gray bars) animals over development. (Two-way ANOVA with a Sidak's post hoc; n=3-4 littermates per genotype per developmental time point). **C.** Quantification of the number of microglia per mm² in *Cx3cl1*^{+/+} (black bars) and *Cx3cl1*^{-/-} (gray bars) frontal cortices across development. (Two-way ANOVA with a Sidak's post hoc; n=3-4 littermates per genotype per developmental time point). **D.** Quantification of vascular area per mm² in *Cx3cl1*^{+/+} (black bars) and *Cx3cl1*^{-/-} (gray bars) mice over development in the frontal cortex. (Two-way ANOVA with a Sidak's post hoc; n=3-4 littermates per genotype per developmental time point). All error bars represent \pm SEM.

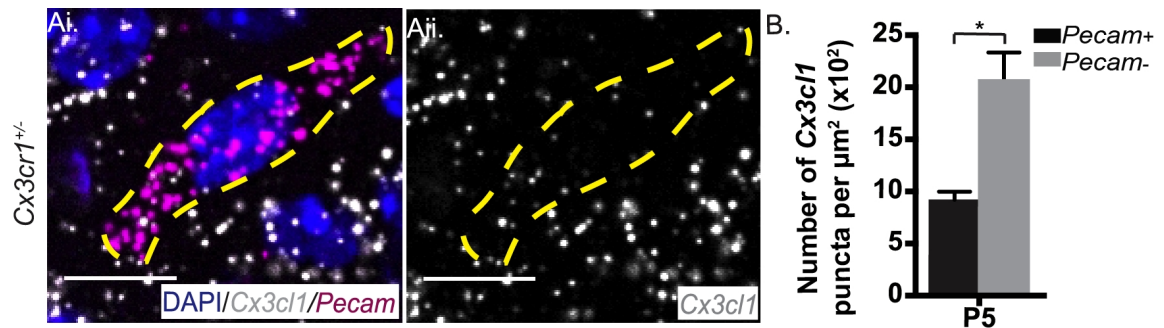


Figure 3.4: *Cx3cl1* is not highly expressed in endothelial cells in the early postnatal brain. **A.** Representative *in situ* hybridization in P5 *Cx3cr1*^{+/-} animals for *Cx3cl1* (grey) and *Pecam* (magenta). The yellow dotted line demarcates the *Pecam* positive signal boundaries used for quantification in B. The *Cx3cl1* channel alone is shown in **Aii**. Scale bar= 10 μm . **B.** Quantification of the number of *Cx3cl1* puncta per μm^2 within the boundaries of the *Pecam*-positive area (black bar) versus *Pecam*-negative cells (gray bars) defined by a boundary, 1.5X the diameter of the nucleus, around the DAPI in P5 *Cx3cr1*^{+/-} frontal cortex. There is significant enrichment of *Cx3cl1* in *Pecam*-negative cells. (Two-tailed paired Student's t-test; n=3 mice; * $p < .05$). All error bars represent \pm SEM.

DISCUSSION

This study provides the first mechanistic insight into how CX3CR1 regulates the timing of microglia-vascular interactions. We have discovered that CX3CR1-dependent timing of microglia-vascular interactions occurs across cortical brain regions during early postnatal development. Moreover, CX3CR1 deficient microglia are less adhesive to cultured endothelial cells when isolated from young, postnatal mice, but not older mice. Finally, we demonstrate that the timing of microglia-vascular interactions is independent of the canonical CX3CR1 ligand, CX3CL1, which is not highly expressed in vascular endothelial cells in the brain during early development. Taken together, these data provide mechanistic insight into microglia-vascular interactions, demonstrating a novel role for CX3CR1 in modulating microglial adhesion to the vasculature and providing evidence that the timing of juxtavascular microglia in the developing brain is independent of CX3CL1. They further suggest a role for CX3CR1-dependent microglial adhesion to blood vessels in regulating the timing of microglial colonization of synapse-dense brain regions.

The timing of microglia-vascular interactions is CX3CR1 dependent but CX3CL1 independent.

CX3CL1 expression in endothelial cells has been implicated in regulating chemo attraction and cell adhesion of peripheral dendritic cells, lymphocytes, leukocytes, Ly6c high monocytes, and THP-1 cells to the vasculature via

CX3CR1 signaling (Muehlhoefer et al. 2000; Umehara et al. 2004; Johnson and Jackson 2013; Goda et al. 2000; Imaizumi, Yoshida, and Satoh 2004; Fong et al. 1998; Hamon et al. 2017; Schwarz et al. 2010; Imai et al. 1997). In contrast, our data demonstrate that, while CX3CR1 regulates the timing of microglia-vascular interactions in the developing cortex, CX3CL1 is not required. This is reminiscent of previous work showing that CX3CR1 can promote adult neurogenesis in the dentate gyrus independent of CX3CL1 (Sellner et al. 2016). The only other proposed ligand of CX3CR1 is CCL26 (Nakayama et al. 2010). However, it is known that CCL26 does not induce chemotaxis of mouse L1.2 cells expressing mouse CX3CR1 (Nakayama et al. 2010), and there is little to no detectable expression of *Ccl26* in the postnatal cerebral cortex (Ye Zhang et al. 2014; Hammond et al. 2019). These data suggest that CCL26 is not a likely candidate to regulate developmental association of microglia with vasculature. Instead, our study raises the intriguing possibility that CX3CR1 is modulating these interactions through a yet-to-be identified ligand derived from the vasculature. Another intriguing possibility is that CX3CR1, a G-protein-coupled receptor (GPCR), could modulate gene expression of cell adhesion molecules, such as integrins, independent of a ligand. GPCRs that lack a ligand, orphaned GPCRs, can participate in intracellular signaling by forming heterodimers with other GPCRs. These interactions can then promote receptor folding, maturation, and/or transport to the cell surface (Marullo and Bouvier 2005; Levoye et al. 2006; X. Tang et al. 2012; Galvez et al. 2001; K. A. Jones et al. 1998; Robbins et

al. 2001). It remains to be determined if CX3CR1 could function similarly in microglia.

Another interesting and related new question raised by our findings is what types of molecules are downstream of CX3CR1 signaling that could regulate vascular adhesion? Our cell adhesion assay demonstrates that microglia isolated from early postnatal *Cx3cr1*^{-/-} mice have reduced adhesion to endothelial cells *in vitro*. This raises the possibility that CX3CR1 signaling could regulate microglial adhesion to the vasculature through modulation of cell adhesion molecules at the cell surface either through transcriptional or post-translational programs. Going forward, it will be important to elucidate downstream CX3CR1 GPCR signaling and whether it is ligand-dependent or -independent.

A possible role for CX3CR1-dependent microglial-vascular adhesion in microglial colonization of synapse-dense brain regions

Our data are intriguing in light of recent work showing microglia are highly juxtavascular as they actively colonize synapse-dense brain regions, the timing of which is dependent on CX3CR1 (Mondo et al. 2020). These transient delays in juxtavascular microglia in CX3CR1 deficient mice are concomitant with delays in recruitment to thalamocortical synapses and are accompanied by delays in synapse maturation (Hoshiko et al. 2012), yet how CX3CR1 coordinates the timing of juxtavascular microglia and colonization remains unknown. Here, we

have uncovered that CX3CR1 deficient microglia isolated from young, but not older, mice are less adhesive to endothelial cells *in vitro*. This developmental difference in endothelial adhesion corresponds to delays in juxtavascular microglia and microglial colonization in CX3CR1 deficient mice (Hoshiko et al. 2012; Mondo et al. 2020). Together, these data are consistent with a model in which microglia adhere to the vasculature in a CX3CR1-dependent manner and use the vasculature as a physical substrate to migrate and localize to the appropriate brain region at the correct time. Inefficient microglial adhesion to the vasculature in young CX3CR1 deficient mice may lead to disruptions to the timing of juxtavascular microglia and subsequent migration and colonization, which is resolved in older animals when CX3CR1 deficient microglia maintain the same adherence levels as control microglia. Indeed, previous work has shown microglia migrate along the vasculature in acute, embryonic and postnatal brain slices as they actively colonize the brain (Smolders et al. 2017; Mondo et al. 2020). Additionally, CX3CR1 deficient microglia display deficits in motility and migration speed (Arnoux and Audinat 2015), however what role the vasculature plays in these motility changes remains unknown.

Microglia-vascular interactions: Implications for CNS disease

Mice that exhibit delays in juxtavascular microglia and microglial colonization during early postnatal periods due to a lack of CX3CR1 also show delays in synaptic maturation and pruning (Paolicelli et al. 2011; Hoshiko et al.

2012; Zhan et al. 2014). Long term, *Cx3cr1*^{-/-} mice have phenotypes associated with ASDs, including decreased functional brain connectivity, deficits in social interactions, and increased repetitive behaviors (Zhan et al. 2014). Thus, CX3CR1-dependent timing of microglia-vascular interactions and subsequent colonization to synaptic regions may be critical for proper neuronal circuit development and defects may be relevant for ASDs. Consistent with this, microglia have been shown to associate with the vasculature in the developing human brain, and individuals with ASDs have alterations in microglial density and synaptic connectivity (Edmonson, Ziats, and Rennert 2016; Morgan et al. 2010). Our work could shed important new mechanistic insight on the role of CX3CR1 in neurological disease. Further understanding of how downstream CX3CR1 signaling is modulates microglia-vascular adhesion in the developing brain will be important to inform disease going forward.

Materials and Methods

Animals

Cx3cr1^{-/-} mice (*Cx3cr1*^{EGFP/EGFP}; stock #005582), *C57Bl6/J* (stock #000664), and *Cx3cr1*^{CreER/CreER} (stock #021160) mice were obtained from Jackson Laboratories (Bar Harbor, ME). *Cx3cl1*^{-/-} mice were provided by Dr. Sergio Lira (Ichan School of Medicine, Mount Sinai). Heterozygous breeder pairs were set up for all experiments and heterozygote littermates were used as controls with equal representation of males and females for each genotype. All experiments were performed in accordance with animal care and use committees and under NIH guidelines for proper animal welfare.

Preparation of tissue for immunofluorescence microscopy

Mice were perfused with 1X Hank's balanced salt solution (HBSS) -magnesium, -calcium, (Gibco, Gaithersburg, MD) prior to brain removal at indicated ages. For analysis of frontal cortex, brains were post-fixed in 4% paraformaldehyde in 0.1M phosphate buffer (PB) for four hours. Brains were placed in 30% sucrose in 0.1M PB and allowed to sink prior to sectioning. Sections were blocked in 10% goat serum, 0.01% TritonX-100 in 0.1M PB for 1 hour before primary immunostaining antibodies were applied overnight. Secondary antibodies were applied for two hours the following day. All steps were carried out at room temperature with agitation. The following antibodies were used: anti-PECAM (Biolegend; San Diego, CA; 1:100).

Confocal microscopy

Immunostained sections were imaged on a Zeiss Observer Spinning Disk Confocal microscope equipped with diode lasers (405nm, 488nm, 594nm, 647nm) and Zen acquisition software (Zeiss; Oberkochen, Germany). For microglia-vascular interaction, microglial density, and vascular density analyses, 20x, single optical plane, tiled images of the frontal cortex were acquired for each animal. To create a field of view (FOV), each tiled image was stitched using Zen acquisition software. Two FOVs (ie. Tiled images) were acquired per animal.

Juxtavascular microglia and microglia density analyses within the frontal cortex

Microglial association with vasculature in the cortex was performed blinded to genotype. Images were analyzed in ImageJ (NIH; Bethesda, MD). Using the DAPI channel as a guide, a region of interest (ROI) was chosen in each cortical layer, I-VI from each 20x stitched tiled image (10 ROIs per animal). The ROI areas were recorded. The same ROI was transposed on the microglial channel and the cell counter ImageJ plugin was used to count the number of microglia in the ROI. The total density of microglia was then calculated by dividing the microglia number by the ROI area. To assess microglial association with the vasculature, the microglia and blood vessel channels were merged and the cell counter plugin was used to manually count the number of microglia with cell

bodies directly apposed to blood vessels. Juxtavascular microglia were defined as microglia with at least 30% of their soma perimeter associated with blood vessels and soma centers that were within 10 μ m of the vessel. The percent of juxtavascular microglia was calculated by summing the total number of microglia associated with vasculature divided by the total number of microglia within the ROI. For each animal, data from the ROIs were averaged together to get a single average per animal for statistical analyses.

Vascular density analysis

Density analysis was performed blinded to genotype from the same tiled and stitched 20x images used for microglia-vascular association analyses. Using ImageJ (NIH; Bethesda, MD) software, the blood vessel channel was thresholded manually and the total blood vessel area was measured. Vascular density was calculated by dividing the blood vessel area by the area of the ROI. For each animal, the vascular density was averaged across all ROI in the two FOV.

Adhesion assay

The adhesion assay was performed as previously described with minor modifications (Lowe and Raj 2015). The day of the assay, cortical microglia were acutely isolated by Percoll gradient. Two mice were perfused with ice-cold 1X HBSS (-magnesium, -calcium) (Gibco) at P3-P5 or P14, cortices of the same age

were dissected and pooled together. Cortices were minced using a razor blade homogenized in a glass homogenizer in RPMI-1640 (Thermo Fisher Scientific). Homogenates were mixed with Percoll (Sigma) to create a 30% percoll and homogenate solution. This solution was layered over a 70% percoll. The homogenate was spun at 500g for 30 minutes at 18 °C with low breaks settings. After centrifugation, myelin debris was removed and microglia were collected from the 70%-30% gradient interphase. Microglia were washed twice in 1X HBSS (-magnesium, -calcium) and centrifuged at 900g for 7 minutes at 4 °C between each wash. After the final wash, microglia were resuspended in HUVEC endothelial cell media, EBM Endothelial Cell Growth Basal Medium (Lonza, Switzerland) supplemented with EGM SingleQuots (Lonza, Switzerland) in a volume that yielded 100,000 microglia for every 200µl. 200µl of resuspended microglia were added to HUVEC endothelial cell line (obtained from Dr. Nathan Lawson at UMMS) plated on coverslips at a density of 50,000-100,000 cells per coverslip. Cells were incubated together for 30-60 minutes at 37 °C. After incubation, coverslips were submersed in 1X phosphate buffered saline (PBS) fifteen times to remove non-adherent cells. Coverslips were then fixed in 4% PFA at room temperature for 15 minutes. Two technical replicates were performed for each experiment and the experiment was repeated four times on separate days (4 biological replicates). Microglial adhesion to HUVEC cells was subsequently quantified similar to previously published work (Lowe and Raj 2015). Single plane 10x epifluorescence images, together with bright field images were collected

using a Zeiss Observer microscope equipped with Zen Blue acquisition software (Zeiss; Oberkochen, Germany). Two images were taken per technical replicate. Images were quantified blinded to genotype in ImageJ (NIH; Bethesda, MD). Microglia and HUVEC cell channels were split. The cell counter plugin was used to count the total number of microglia in a 10x FOV. The microglia and HUVEC channel were then merged together and the cell counter plugin was used to count the number of microglia that were adherent to HUVECs. This number was then divided by the total number of microglia within the FOV to calculate the percent of microglia adherent to HUVECs. For each biological replicate, data were averaged over the two technical replicates prior to statistical analyses.

***In situ* RNA hybridization**

In situ RNA hybridization was performed according to the manufacturer's specification with slight modifications (ACDBio; Newark, CA). Briefly, mice were perfused with 4% PFA and brains were post-fixed for 24 hours. 10 μ m cryosections were prepared, equilibrated to room temperature for 1 hour, and then washed in 1X PBS for 5 minutes. Sections were treated with "Protease III" for 20 minutes at 40 °C and rinsed with RNase free water. *In situ* probes were added and incubated for 2 hours at 40 °C. Subsequent amplification steps were performed according to the manufacturer's specification. *In situ* sections were then imaged on a Zeiss Observer Spinning Disk Confocal microscope and Zen acquisition software (Zeiss; Oberkochen, Germany). For each animal, twelve 63x

fields of view were acquired in the frontal cortex with 25-35 z-stacks at 0.22 μ m spacing. Images were analyzed using ImageJ (NIH; Bethesda, MD). Z-stacks were opened, one optical plane was chosen for analysis, and the *Pecam* and *Cx3cl1* channels were split. To quantify the number of puncta in *Pecam* positive area, the free hand selection tool was used to draw a ROI around the *Pecam* positive area. The area of the *Pecam* positive ROI was recorded. The *Cx3cl1* channel was manually thresholded, the *Pecam* positive ROI was superimposed on the *Cx3cl1* channel, and the number of *Cx3cl1* puncta within the *Pecam* positive area was recorded. To quantify the number of puncta in a *Pecam* negative area, a circle that was 1.5x the diameter of a *Pecam* negative nucleus was drawn. Nuclei smaller than 5 μ m were excluded. The area of the circle was recorded, superimposed on the *Cx3cl1* channel, and the number of *Cx3cl1* puncta within the ROI was recorded. To calculate the number of *Cx3cl1* puncta per μm^2 ($\times 10^2$), the number of *Cx3cl1* puncta in the *Pecam* positive area was divided by the *Pecam* positive ROI or the *Pecam* negative ROI and multiplied, respectively, by one hundred. Fifteen cells per animals were analyzed and data for each animal was averaged across all twelve fields of view prior to statistical analyses.

Statistical analysis

GraphPad Prism 7 (La Jolla, CA) provided the platform for all statistical and graphical analyses. The ESD method was run for each ROI per animal to identify

outliers. Significant outliers were removed prior to analyses. Analyses included Students t-test when comparing 2 conditions or one-way ANOVA followed by Dunnett's post hoc analysis or two-way ANOVA followed by Sidak's or Tukey's post hoc analyses (indicated in figure legends).

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Chapter IV

Discussion

Over the past 20 years, there has been a growing appreciation for the importance of microglia in the healthy CNS. In contrast to classically held beliefs, seminal imaging studies revealed that “resting” microglia in the healthy brain are highly active, constantly extending and retracting their processes which are in frequent contact with neurons, synapses, and other glial cells (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005; Schafer et al. 2012; Tremblay, Lowery, and Majewska 2010; Frost and Schafer 2016). These descriptions of interactions between microglia and other resident CNS cell types has now led to a new understanding that microglia are important for neural circuit structure and function and has raised questions about what other CNS cell types microglia contact (Schafer et al. 2012; Tremblay, Lowery, and Majewska 2010; Paolicelli et al. 2011; Gunner et al. 2019). Microglia are known to contact the brain vasculature, however, the vast majority of these studies have been in the context of disease (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018). In this thesis, I investigate the developmental timing, dynamics, localization, and molecular mechanisms of juxtavascular microglia in the healthy postnatal CNS. In Chapter II, I demonstrate that microglia are highly associated with the vasculature during the first week of postnatal development, the timing of which is dependent on the fractalkine receptor (CX3CR1). Moreover, I find that microglial association with the vasculature is concomitant with a peak in microglial

colonization of cortex and synapse-dense brain regions. As microglia colonize the cortex, juxtavascular microglia are highly motile along vessels and become largely stationary in adulthood. This change in microglia motility occurs as astrocyte endfeet arrive on the vasculature. Finally, I determine that juxtavascular microglia associate with both the basal lamina and astrocyte endfeet surrounding the vasculature through development and adulthood. In Chapter III, I investigate the mechanism by which CX3CR1 regulates the timing of microglia-vascular interactions. I determine that CX3CR1 modulates the timing of juxtavascular microglia throughout the developing cortex. Moreover, I demonstrate that CX3CR1 deficient microglia isolated from young mice are less adhesive to endothelial cells. However, surprisingly I find that the timing of microglia-vascular interactions is independent of CX3CL1, the canonical ligand to CX3CR1. Together, this work provides the first extensive analysis of juxtavascular microglia in the healthy, developing and adult brain and lays the fundamental groundwork to investigate the function of these cells in the healthy and diseased brain.

Part I. Microglial association and migration along the vasculature in the developing brain: implications for microglial colonization.

Microglia are born as primitive macrophages in the embryonic yolk sac and enter the neuroepithelium at embryonic day E9.5 by crossing the pial surface and lateral ventricles (Navascués et al. 2000; Swinnen et al. 2013; Ginhoux et al.

2010). They then migrate and proliferate to colonize the embryonic brain in a rostral-to-caudal gradient (Sorokin et al. 1992; Navascués et al. 2000; Swinnen et al. 2013; Alliot, Godin, and Pessac 1999; Perry, Hume, and Gordon 1985; Ashwell 1991). While signaling mechanisms have been identified to regulate the initial infiltration of microglial precursors into the brain parenchyma (Ginhoux et al. 2010; Arno et al. 2014; Ueno and Yamashita 2014), far less is known about the mechanisms regulating microglial localization to the appropriate brain regions during postnatal development. In this thesis, I have identified that microglia are highly juxtavascular and migratory on vessels during the first postnatal week, a time of active microglial colonization of the cortex and synapse-dense brain regions. Moreover, I demonstrate that the timing of these interactions is dependent on CX3CR1, a molecule implicated in regulating recruitment of microglia into synapse-dense brain regions (Paolicelli et al. 2011; Hoshiko et al. 2012). Together, these data suggest microglia utilize blood vessels to colonize the developing brain and raises intriguing future directions including understanding the role of the vasculature as a scaffold for microglial migration, investigating CX3CR1-dependent timing of juxtavascular microglia, and exploring possible mechanisms regulating juxtavascular microglia migration directionality and colonization. Understanding how microglia localize to the appropriate brain region at the correct time will provide insight into neurodevelopmental disorders, such as autism spectrum disorders (ASDs), that are characterized by alterations

in microglial density and synaptic changes (Edmonson, Ziats, and Rennert 2016; Morgan et al. 2010).

The vasculature as a scaffold for microglial colonization

In the developing brain, the vasculature serves as a scaffold for the migration of several different cell types. During embryonic development, gamma-aminobutyric acid (GABA) inhibitory interneurons are associated with blood vessels as they migrate towards the cortex (Won et al. 2013; Barber et al. 2018; Li et al. 2018). Additionally, the vasculature serves as a scaffold for migration of another glial subtype in the brain, oligodendrocyte precursor cells (OPCs). In the developing mouse forebrain, the first OPCs originate from ventral regions of the medial ganglionic eminence and the anterior entopeduncular area at E12 and become highly associated with, and migrate on, vascular networks (Kessaris et al. 2006; Tsai et al. 2016). OPC migration and localization is disrupted in mice with defective vascular architecture, suggesting that physical interactions with the vascular endothelium are required for OPC migration (Tsai et al. 2016). Previous work has shown microglia can migrate along the vasculature in acute embryonic brain slices and brain slices prepared from postnatal mice in an injury context (Smolders et al. 2017; Grossmann et al. 2002). In Chapter II, I demonstrate that microglia are highly associated with and migrate on vasculature during the first week of postnatal development, a time of active microglial colonization.

Together, these data raise the intriguing possibility that microglia utilize the vasculature as a scaffold to migrate to the appropriate brain region in the developing brain, similar to GABA inhibitory neurons and OPCs, however future experiments are required. An ideal experiment is to assess microglial colonization *in vivo* in a mouse model with defective vascular architecture, such as the G protein-coupled receptor 124 (GPR124) knockout mice used to study OPC-vascular migration (Tsai et al. 2016). However, mice with disrupted vasculature are embryonic lethal (Sohet and Daneman 2013), thus vascular-dependent microglial colonization would have to be assessed in the context of embryonic development. Alternatively, genes required for appropriate vascular structure could be conditionally removed during postnatal development using a Cre-Lox system, but these conditional knockouts can lead to a leaky blood-brain-barrier (BBB) (Sohet and Daneman 2013), which attract microglia to the vasculature and could make interpreting the results difficult (Davalos et al. 2012; Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018). Novel techniques to disrupt vascular architecture without embryonic lethality or disrupting BBB permeability will help to overcome obstacles in addressing this future direction.

CX3CR1-dependent timing of juxtavascular microglia and colonization

CX3CR1, a G-protein-coupled chemokine receptor (GPCR) that is highly enriched in microglia (Combadiere, Ahuja, and Murphy 1995; Raport et al. 1995), has been implicated in regulating microglial colonization of synapse-dense brain

regions. Mice deficient in CX3CR1 (*Cx3cr1*^{-/-}) have delays in their localization to synapse-dense brain regions such as the barrel cortex and hippocampus, resulting in delays in synaptic development. However, how CX3CR1 coordinates colonization is unknown (Paolicelli et al. 2011; Zhan et al. 2014; Hoshiko et al. 2012). In Chapter II, I demonstrate that microglia are highly associated with and migrate on vessels during times of active colonization. The timing of microglia-vascular interactions is delayed in *Cx3cr1*^{-/-} mice concomitant with delays in microglia localization to synapse-dense brain regions. These data raise the intriguing possibility that CX3CR1 regulates colonization through microglia-vascular interactions.

One possible mechanism by which this may occur is through CX3CR1-dependent microglial adhesion to the vasculature. In the periphery CX3CR1 mediates adhesion of peripheral immune cells, such as lymphocytes, leukocytes, Ly6c high monocytes, and THP-1 cells, to the vasculature through binding to CX3CL1, the only known *in vivo* ligand to CX3CR1 (Muehlhoefer et al. 2000; Umehara et al. 2004; Johnson and Jackson 2013; Goda et al. 2000; Imaizumi, Yoshida, and Satoh 2004; Fong et al. 1998; Hamon et al. 2017; Schwarz et al. 2010; Imai et al. 1997). To this end, in Chapter III I demonstrate that *Cx3cr1*^{-/-} microglia isolated from young mice (P3-5) are less adhesive to cultured endothelial cells compared to control mice, suggesting CX3CR1 regulates microglial adhesion to the vasculature *in vivo*. However, surprisingly, I find that the timing of microglia-vascular interactions *in vivo* is independent of CX3CL1,

raising questions of how CX3CR1 regulates adhesion to the vasculature independent of CX3CL1. One possibility is that deletion of CX3CR1, a GPCR, could lead to alterations in microglial gene expression that affect microglial adhesion to the vasculature. Previously work has demonstrated that other functions of CX3CR1, such as promoting adult neurogenesis of the dentate gyrus, are independent of CX3CL1 and dependent on downstream CX3CR1 signaling (Sellner et al. 2016). Here, they report *Cx3cr1*^{-/-} mice have altered gene expression compared to wild type (WT) mice and identify changes in sirtuin 1 (SIRT1)/P65 expression as being responsible for the change in the number of newborn and proliferative cells in the subgranular zone observed in *Cx3cr1*^{-/-} mice (Sellner et al. 2016). Single cell RNA-sequencing (RNA-seq) published from the Schafer lab demonstrates that *Cx3cr1*^{-/-} mice also have altered expression of molecules with known adhesion roles, such as osteopontin (SPP1), during early postnatal development (P5) (Gunner et al. 2019). Whether altered expression of cell adhesion molecules in *Cx3cr1*^{-/-} mice is responsible for reduced adhesion to the vasculature and delays in microglial colonization, independent of CX3CL1, remains an open question. Future experiments include assessing microglia-vascular interactions, juxtavascular migration, and microglial colonization in knockout models of candidate molecules identified by the Schafer lab RNA-seq data set. Additionally, RNA-seq of *Cx3cr1*^{-/-} and WT control mice at multiple developmental time points, as microglia colonize the brain, may reveal candidate genes important for microglial adhesion to the vasculature. An ideal candidate

would be a cell adhesion molecule that is highly expressed in microglia during embryonic and the first week of postnatal development, that is significantly down regulated in *Cx3cr1*^{-/-} mice.

Another way CX3CR1-dependent timing of juxtavascular microglia could regulate colonization is through modulating microglia migration and motility. Previous work indicates that *Cx3cr1*^{-/-} microglia have reduced migration and motility. In response to a laser ablation in the retina, *Cx3cr1*^{-/-} mice have significantly slower migratory response to the injury site (Liang et al. 2009). Moreover, 2-photon live imaging studies in the P5-P9 barrel cortex demonstrate that *Cx3cr1*^{-/-} microglia have reduced soma velocity in response to a chemoattractant cue, a P2RY12 agonist (Arnoux and Audinat 2015). These data support a model in which *Cx3cr1*^{-/-} microglia have slower migratory speed, resulting in a delayed association with the vasculature and delayed microglial colonization. However, further experiments are required to test this model. 2-photon live imaging of the somatosensory cortex in *Cx3cr1*^{-/-} mice during peak microglial colonization, between P5 and P9, are required to assess *Cx3cr1*^{-/-} microglia migration velocity towards, and while associated with, the vasculature.

Another question raised by this work is, how does CX3CR1 signal to regulate microglia vascular interactions? One possibility is through down stream GPCR pathways. CX3CR1 is a GPCR that signals through the G_q, in turn activating phosphatidylinositol-3 (PI-3) kinase pathway (Lyons et al. 2009; Imai et al. 1997). As PI-3 kinase has been implicated in regulating cell motility and

chemotaxis (Kölsch, Charest, and Firtel 2008), deletion of CX3CR1 may lead to alterations in downstream PI-3 kinase signaling and subsequent changes in microglial motility and chemotaxis that could delay microglial localization to the vasculature. Alternatively, CX3CR1 has been shown to mediate adhesion independent of G protein signaling, but required the architecture of a chemokine domain atop the mucin stalk (Imai et al. 1997). Thus, CX3CR1 could also signal as an adhesion molecule through an extracellular domain function to mediate the timing of microglia-vascular interactions. Finally, CX3CR1 could signal through a scaffold/heterodimer protein-protein interaction with a second receptor. To determine how CX3CR1 signals to mediate the timing of juxtavascular microglia, different domains on CX3CR1 could be mutated such that downstream GPCR signaling can't occur, CX3CR1 cannot bind to a ligand through the ligand-binding domain, or CX3CR1 cannot adhere by mutating the chemokine domain. The timing of juxtavascular microglia could then be assessed within each of these conditions.

Understanding how CX3CR1 regulates the timing of juxtavascular microglia and colonization is important given that *Cx3cr1*^{-/-} mice also show delays in synaptic maturation and pruning (Paolicelli et al. 2011; Hoshiko et al. 2012; Zhan et al. 2014). Long term, *Cx3cr1*^{-/-} mice have phenotypes associated with ASDs, including decreased functional brain connectivity, deficits in social interactions, and increased repetitive behaviors (Zhan et al. 2014). Thus, CX3CR1-dependent timing of juxtavascular microglia and microglial colonization

to synaptic regions may be critical for proper neuronal circuit development and defects may be relevant for ASDs. The work presented in this thesis could shed important new mechanistic insight on the role of CX3CR1 in neurological disease. Further dissection of how CX3CR1 signaling is modulating microglia-vascular interactions in the developing brain, as well as in models of ASDs, will be important to inform disease going forward.

Possible mechanisms regulating juxtavascular microglia migration directionality and colonization

In rodents, signaling mechanisms have been identified to regulate the initial infiltration of microglial precursors into the brain parenchyma, such as matrix metalloproteinases (MMPs), stromal cell derived factor 1 (SDF-1), and Cxcl12/Cxcr4 signaling (Ginhoux et al. 2010; Arno et al. 2014; Ueno and Yamashita 2014). However the mechanism underlying microglial migration directionality and colonization of the correct brain region in such a precise time window is an unresolved question. In this thesis, I demonstrate that juxtavascular microglia migrate on blood vessels as they colonize the brain and this migration becomes restricted in adulthood, when colonization is complete. Moreover, I find that in early postnatal development, juxtavascular microglia have a motility trajectory within 15° of the vasculature, demonstrating directional migration along vessels. Finally, I provide evidence that the timing of microglia-vascular interactions is dependent on CX3CR1, suggesting CX3CR1-CX3CL1 signaling

may serve as a migration and colonization cue for juxtavascular microglia. CX3CL1 is unique amongst chemokines because it is synthesized as a transmembrane molecule, but can be cleaved into a soluble form that contains the chemokine domain that serves as a chemoattractant signal (Umehara et al. 2004; B. Jones, Beamer, and Ahmed 2010). In Chapter III, I find that *Cx3cl1* is highly enriched outside of endothelial cells, likely in neurons (Gunner et al. 2019), suggesting delays in microglia-vascular association in *Cx3cr1^{-/-}* mice may be due to disruptions in chemokine gradient signaling from neuronal sources of soluble CX3CL1 versus a direct effect of vascular adhesion. Arguing against this hypothesis, I demonstrate that the timing of microglia-vascular interactions is CX3CL1 independent. While CX3CR1 may regulate juxtavascular microglia adhesion and/or motility through an alternative, CX3CL1 independent method (discussed above in “*CX3CR1-dependent timing of juxtavascular microglia and colonization*”), these data raise the intriguing possibility of an unidentified cue modulating the directionality of juxtavascular microglia migration and colonization.

Clues to different signals that could regulate juxtavascular microglia migration and colonization can be taken from the literature. As described above, several different cell types utilize the vasculature to migrate in the healthy brain. The developing vasculature regulates the migration of interneurons via diffusible signals, such as GABA and vascular endothelial growth factor (VEGF) secreted by vascular endothelial cells (Won et al. 2013; Barber et al. 2018; Li et al. 2018;

Fujioka, Kaneko, and Sawamoto 2019). Interestingly, microglia express VEGF receptor 1 (VEGFR-1) and it plays a role in microglia chemotactic response induced by amyloid- β peptide (Y Zhang et al. 2014; Ryu et al. 2009). In the adult brain, neuroblasts born in the subventricular zone migrate along blood vessels to the olfactory bulb, which is regulated by endothelial cell derived brain-derived neurotrophic factor (BDNF) and neurotrophin receptor P75 (P75NTR) expression on migrating neuroblasts (Snapyan et al. 2009). Interestingly, neuroblasts derived GABA release induces Ca^{2+} -dependent tyrosine receptor kinase β (Trk β) (a potent BDNF receptor) insertion in astrocytes, suggesting astrocytes can limit the availability of BDNF and thus also play a role in neuroblast migration (Snapyan et al. 2009). Given my findings in Chapter II that inhibition of juxtavascular microglia migration coincides with the appearance of astrocyte endfeet on the vasculature, the idea of a tripartite regulation of microglial migration on vasculature and colonization is intriguing.

Another candidate mechanism that could regulate the directionality of juxtavascular microglia migration and colonization is neuronal activity. Sensory-related changes in neuronal activity regulate the structure of vascular networks in the cortex (Lacoste et al. 2014). Increases in neuronal activity in the somatosensory barrel cortex, through whisker stimulation, lead to increases in vascular density and branching while dampening neuronal activity results in decreased vascular density and branching (Lacoste et al. 2014). In addition to changes in vascular structure, changes in neuronal activity modulate microglia

process dynamics, with microglia moving towards the source of neuronal activity (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005; Liu et al. 2019; Stowell et al. 2019; Tremblay, Lowery, and Majewska 2010). One pathway that could regulate neuronal activity dependent juxtavascular migration is ATP-P2RY12 signaling. P2RY12 is a purinergic receptor expressed exclusively by microglia in the CNS that regulates microglial dynamics in response to ATP (Butovsky et al. 2014; Haynes et al. 2006). Juxtavascular microglia require P2RY12 to extend their processes towards an injured vessel (Lou et al. 2016). Moreover, activated neurons release ATP, which has been shown to mediate microglia process outgrowth (Dissing-Olesen et al. 2014). Together, these data suggest neuronal activity could act as a chemoattractive cue for juxtavascular microglia migration through ATP-P2RY12 signaling.

While thought provoking, future experiments are required to test these hypotheses. First, single-cell RNA-seq experiments could be performed to identify candidate molecules that regulate microglial chemotaxis. Based on previously published literature, these molecules will likely be expressed by vascular cells, but could also be expressed by neurons. Once candidate molecules are identified, juxtavascular microglial association, migration, and colonization can be assessed if knockout mouse models exist. Second, to determine if neuronal activity plays a role in the directional migration and colonization of juxtavascular microglia, a whisker deprivation paradigm can be used to dampen neuronal activity in the barrel cortex and localization of

juxtavascular microglia can be assessed in the absence of neuronal activity (Lacoste et al. 2014; Gunner et al. 2019). Finally, to determine if ATP-P2RY12 signaling plays a role in juxtavascular microglia migration, microglia-vascular associations and juxtavascular migration can be assessed in P2RY12 knockout mice. Identifying mechanisms that regulate juxtavascular migration directionality will be key to determine the relative importance of microglia-vascular interactions for microglial colonization, brain development, and long-term CNS function.

Concluding remarks

In this thesis, I have demonstrated that microglia are highly associated with the vasculature concomitant with colonization of the cortex and synapse-dense brain regions. I show that microglia are migratory on vasculature as they colonize the cortex and stationary in adulthood when colonization is complete. Finally, I show that the timing of these interactions is dependent on CX3CR1, a molecule known to regulate microglial colonization of synapse-dense brain regions (Paolicelli et al. 2011; Hoshiko et al. 2012). Together, these data suggest microglia utilize the vasculature as a scaffold to migrate on and colonize the appropriate brain region at the correct time. Ultimately, these studies linking juxtavascular microglia migration and colonization are correlations. Future experiments aimed at determining if the vasculature serves as a scaffold for microglial colonization, determining how CX3CR1 regulates the timing of juxtavascular microglia independent of CX3CL1, and identifying mechanisms

regulating juxtavascular microglia directionality will provide further insight regarding if and how microglia utilize the vasculature for colonization. Understanding how microglia localize to the appropriate brain region at the correct time, and the role juxtavascular microglia play in this colonization is important given that microglia have been reported to be localized to the vasculature in the human brain and several neurodevelopmental disorders, such as ASDs, are characterized by alterations in microglial density (Edmonson, Ziats, and Rennert 2016; Morgan et al. 2010).

Part II. Juxtavascular microglia-astrocyte interactions and crosstalk at the neurovascular unit.

At first glance, microglia and astrocytes appear to be very different cell types. Astrocytes are derived from neuroepithelial progenitors, are tissue embedded, and are non-motile, whereas microglia are derived from a hematopoietic common myeloid progenitor that enters the brain during embryonic development and have highly dynamic processes (Molofsky and Deneen 2015; Ginhoux et al. 2010; Allen and Eroglu 2017; Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005). Despite their differences, microglia and astrocytes have the ability to communicate and coordinate their functions both in the healthy and diseased brain (Vainchtein and Molofsky 2020). For example, both microglia and astrocytes are found in close association at the synapse and play complementary roles in support of synapse formation and remodeling (Allen

and Eroglu 2017; Clarke and Barres 2013; Tremblay, Lowery, and Majewska 2010; Schafer et al. 2012; Vainchtein and Molofsky 2020). Along with synapses, astrocytes processes, termed endfeet, are found at the vasculature and play an integral role in the maturation and maintenance of the blood brain barrier (BBB) (Abbott, Rönnbäck, and Hansson 2006; Kimelberg and Nedergaard 2010; Macvicar and Newman 2015). In this thesis, I demonstrate that microglia are also localized to the vasculature and are in direct contact with both astrocyte endfeet and the basal lamina throughout development into adulthood. Together, these data raise several questions: 1.) Do astrocytes play a role in regulating juxtavascular microglial association and migration? 2.) Do juxtavascular microglia and astrocytes communicate at the vasculature to coordinate vascular function? 3.) Could the vasculature serve as novel model to study microglia-astrocyte crosstalk? Given that both astrocytes and microglia play important roles in the maintenance and dysfunction of the BBB in the injured and diseased brain (Eilam et al. 2018; Michinaga and Koyama 2019; Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018; Haruwaka et al. 2019), understanding microglia-astrocyte interactions and crosstalk at the NVU may provide insight into neurodegenerative diseases.

A potential role for astrocyte endfeet in juxtavascular microglial association and migration

Astrocytes are a key cellular component of the NVU that are born and begin extending their processes to form endfeet along blood vessels during the first postnatal week (Daneman et al. 2010). These astrocyte endfeet ultimately surround and ensheath 60-95% of vasculature by adulthood (Mathiisen et al. 2010; Korogod, Petersen, and Knott 2015). During this time when vessels are left uncovered by astrocyte endfeet, I find that a high percentage of microglia are associated with and migrate on the vasculature. Interestingly, I demonstrate that the decline in the percent of microglia associated with the vasculature and juxtavascular microglia migration corresponds with expansion of astrocyte endfeet territory on vessels over development. Together, these data raise the interesting possibility that expansion of astrocyte endfeet territory on blood vessels excludes microglia from associating with and migrating on the vasculature during early postnatal development. One mechanism by which this may occur is by astrocytes releasing signals that inhibit juxtavascular microglia migration. Indeed, *in vitro* work suggests that astrocytes can inhibit the migration of Schwann cells, glial cells found in the peripheral nervous system (Afshari, Kwok, and Fawcett 2010; Afshari et al. 2010). In addition, time-lapse imaging demonstrates that reactive astrocytes can inhibit neuroblast migration towards a lesion caused by a stroke (Kaneko et al. 2018). Whether similar mechanisms regulate juxtavascular migration is unknown and is an important future direction.

Alternatively, astrocyte endfeet expansion could exclude juxtavascular microglial association and migration on vasculature by simply limiting the

vascular surface area available for microglia to contact. In Chapter II, I demonstrate that across all developmental ages juxtavascular microglia are in direct contact with the basal lamina, a complex of extracellular matrix proteins that could provide an adhesive substrate for microglial association and migration (Thomsen, Routhe, and Moos 2017). Therefore, as astrocyte endfeet expand during early postnatal development, they may limit the basal lamina surface area available for microglia to associate with, thus causing juxtavascular microglia dissociation and inhibiting migration. Along these same lines, the expansion of astrocyte endfeet territory on vessels may physically remove juxtavascular microglia from the vasculature, leading to decreases in the percent of microglia on vasculature.

Given the complementary role that microglia and astrocytes play in regulating each other's function, another interesting direction is to determine if juxtavascular microglia contribute to astrocyte endfeet expansion on vessels during development. This could be in a positive manner, such as promoting astrocyte endfeet expansion by releasing chemoattractive signals. Indeed, microglia release soluble factors that can direct the migration of neuronal precursor cells in the subventricular zone (Aarum et al. 2003; Xavier et al. 2015). However, it could be in a negative manner, microglia may express a repellent cue that inhibits astrocyte endfeet from ensheathing vessels during the first postnatal week, which is down regulated in older animals. Answers to these

questions will provide deeper insight into the relationship between juxtavascular microglia and astrocyte endfeet.

In the future, several experiments will be required to test these hypotheses. First, live imaging of astrocyte endfeet-juxtavascular microglia interactions on blood vessels through several different developmental time points will be critical to determine the role of astrocyte endfeet expansion in juxtavascular microglia association and migration. Additionally, assessing juxtavascular microglial association and migration in a mouse model with delayed astrocyte endfeet, such as fibroblast growth factor 2 (FGF2) knock out mice (Saunders et al. 2016), will provide clues to the role of astrocyte endfeet in juxtavascular microglia dynamics. Finally, to determine if microglia contribute to astrocyte endfeet expansion during development, endfeet coverage on blood vessels can be quantified in mice lacking microglia, such as CSF1R KO mice (Ginhoux et al. 2010; Erlich et al. 2011). To determine if juxtavascular microglia regulated coverage in adulthood, a CSF1R inhibitor, PLX3397 (Elmore et al. 2014), can be used to deplete microglia and astrocyte endfeet coverage on vessels can be assessed. These experiments will provide further insight into microglia-astrocyte interactions at the vasculature and will be key to determine the relative importance of astrocyte endfeet juxtavascular microglia dynamics.

Could juxtavascular microglia-astrocyte crosstalk coordinate vascular function?

The cells that make up the NVU, including endothelial cells, pericytes, smooth muscle cells, pericytes, the basal lamina, astrocytes, and neurons, work in concert to ensure proper function of the vasculature and the BBB (Daneman and Prat 2015; Mastorakos and McGavern 2019; Alvarez, Katayama, and Prat 2013). In particular, astrocytes play a key role in promoting and maintaining BBB and vascular function by communicating between cells of the NVU. For example, astrocytes provide a cellular link between neuronal circuitry and blood vessels, as astrocytes relay signals that regulate blood flow in response to neuronal activity (Attwell et al. 2011; Gordon, Howarth, and Macvicar 2010). This includes regulating the contraction/dilation of vascular smooth muscle cells surrounding arterioles as well as pericytes surrounding capillaries (Daneman and Prat 2015). In addition, astrocytes are known to produce an array of factors that can modulate endothelial function by inducing expression of junctional proteins, altering the permeability of the BBB (Wang et al. 2008; Alvarez et al. 2011; Alvarez, Katayama, and Prat 2013; Daneman and Prat 2015). Finally, astrocytic extracellular matrix proteins have been shown to regulate pericyte differentiation and smooth muscle cell function, which helps to maintain BBB integrity (Z. Chen et al. 2013; Yao et al. 2014). In this thesis, I demonstrate for the first time that microglia are apart of the NVU, with their somas directly contacting the basal lamina and astrocyte endfeet throughout the lifetime of animal. This raises the question, could juxtavascular microglia-astrocyte communication regulate vascular function?

One mechanism by which astrocytes communicate with the NVU is through cytokine signaling. Cytokines released by reactive astrocytes in close proximity to the BBB induce tight junction re-organization through tumor necrosis factor (TNF), interferon (IFN)- γ , and CCL2 (Chaitanya et al. 2011; Yao and Tsirka 2014). Moreover, astrocyte-derived IL-6 decreases endothelial cell barrier function *in vitro* (Takeshita et al. 2017). In addition to cross talk with the NVU, cytokines are also an important mechanism of astrocyte-microglia crosstalk (Vainchtein and Molofsky 2020). For example, astrocyte-derived IL-33 can promote microglial synapse engulfment and neuronal circuit development, while microglia-conditioned media increases the differentiation of NPCs into astrocytes through IL-6 (Vainchtein et al. 2018; Nakanishi et al. 2007). Together, these data provide a foundation to explore if astrocyte-juxtavascular microglia crosstalk through cytokine signaling could have consequences on vascular development and integrity. In the future, experiments such as *in vitro* microglia, astrocyte, and endothelial co-cultures will be necessary to determine if microglia or astrocyte derived cytokines work in synchrony to modulate endothelial cellular function.

Another potential mediator of astrocyte-microglia crosstalk at the vasculature is the extracellular matrix (ECM). The parenchymal basal lamina is comprised of ECM proteins, such as laminin, secreted by astrocyte endfeet and is the site of juxtavascular microglia contact with the vasculature (Daneman and Prat 2015; Thomsen, Routhe, and Moos 2017; Mondo et al. 2020). Astrocytic extracellular matrix proteins have been implicated in regulating BBB

development, function, and permeability. For example, knockout of laminin $\alpha 2$ chain (LAMA2^{-/-}) expressed by astrocytes and pericytes causes infiltration of inflammatory cells in the brain parenchyma, changed organization of tight junction proteins, reduced pericyte coverage, and extravasation of albumin (Menezes et al. 2014). Astrocytic laminin also affects pericyte differentiation by maintaining the pericytes in non-contractile state, which stabilizes BBB integrity (Yao et al. 2014). While the exact extracellular matrix proteins expressed by juxtavascular microglia is not known, microglia do express MMPs, a group of enzymes that are responsible for the degradation of most ECM proteins, which disrupt BBB function (Rosenberg 1995). Specifically, microglia express MMP-2 and MMP-9, which have been shown to disrupt the parenchymal basal lamina in a mouse model of multiple sclerosis (Kieseier et al. 1998; Dubois et al. 1999; Romanic and Madri 1994; Könnecke and Bechmann 2013). Thus, MMPs secreted by juxtavascular microglia at the basal lamina may control the ECM composition secreted by astrocytes, leading to functional changes in the vasculature. Additionally, recent work demonstrates that microglia can engulf ECM proteins in an IL-33 dependent manner (Nguyen et al. 2020), raising the possibility that juxtavascular microglia could engulf basal lamina ECM, influencing astrocytes and vascular function. Although these models of juxtavascular microglia-ECM-astrocyte communication may be indirect, they would still require the two cell types to create a functional change at the vasculature. Future experiments include identifying ECM proteins and MMPs that

are expressed by juxtavascular microglia. Methodologies to identify juxtavascular microglia specific genes will be discussed in Part IV of the discussion.

The NVU: a novel model to investigate microglia-astrocyte crosstalk

Although microglia and astrocytes perform complementary functions in neuronal development and homeostasis, few studies have addressed how microglia and astrocytes communicate to coordinate these functions. Previous work has demonstrated that microglia and astrocyte processes are both found at synapses and can promote developmental synapse formation and pruning (Allen and Eroglu 2017; Clarke and Barres 2013; Tremblay, Lowery, and Majewska 2010; Schafer et al. 2012). One study found that astrocytes can secrete transforming growth factor β (TGF- β) that may positively regulate complement component 1q (C1q) expression and promote microglial phagocytosis (Bialas and Stevens 2013). Additionally, it has been shown that IL-33 produced by developing astrocytes directly increased microglial phagocytic ability (Vainchtein et al. 2018). Beyond this work, few studies have shown direct evidence of microglia-astrocyte crosstalk. A novel model to study microglia-astrocyte crosstalk could provide new avenues to investigate molecular mechanisms of communication and functional outcomes. In this thesis, I find that juxtavascular microglia are in direct contact with astrocytes at the vasculature across all developmental time points, providing a novel modality to investigate microglia-astrocyte crosstalk. Questions aimed at understanding what molecules microglia

and astrocytes use to communicate, how microglia and astrocytes physically interact, how microglial dynamics are affected by astrocytes, and the functional outcomes of these interactions can be explored here.

Concluding remarks

In this thesis, I demonstrate that microglia are localized to the vasculature and are in direct contact with both astrocyte endfeet and the basal lamina throughout development into adulthood. Additionally, I show that changes in the percent of juxtavascular microglia and juxtavascular migration correlate with an expansion in astrocyte endfeet, raising the possibility that microglia and astrocytes are able to communicate and regulate one another at the vasculature. This work lays the foundation to investigate microglia-astrocyte crosstalk, an understudied and important topic in glial biology. An interesting direction raised by this thesis to understand if astrocytes play a role in regulating juxtavascular microglial association and migration, which will provide key insights into mechanisms regulating microglial colonization as well as mechanisms by which microglia and astrocytes communicate. Another future direction of this thesis is to determine if juxtavascular microglia and astrocytes communicate at the vasculature to coordinate vascular function. Vascular dysfunction, initiated and perpetuated by microglia and astrocytes, is characteristic of several neurological disorders (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018; Vainchtein and Molofsky 2020), thus understanding mechanisms of juxtavascular microglia-

astrocyte crosstalk could provide novel therapeutic targets to treat these disorders.

Part III. Juxtavascular microglia-vascular crosstalk and functions in the healthy CNS

Juxtavascular microglia were first identified in the rodent brain in 1991 (Ashwell 1991; Lassmann et al. 1991). Using ultrastructural analysis, the authors demonstrate that microglial processes contact the vascular basal lamina between astrocyte endfeet (Lassmann et al. 1991). Since this initial description, few studies have focused on these cells in the healthy brain. In this thesis, I report the first extensive analysis of juxtavascular microglia in the healthy, developing and adult brain. I demonstrate that microglia-vascular interactions are developmentally regulated, with a high percentage of microglia juxtavascular during the first week of postnatal development. Moreover, I demonstrate that microglial cell bodies directly contact the basal lamina and astrocyte endfeet throughout development and adulthood. These data lay the fundamental groundwork to gain a deeper understanding of juxtavascular microglia regulation of the vasculature, vascular regulation of juxtavascular microglia, and potential roles of microglia at the vasculature. With new evidence that microglia could be a conduit by which changes in peripheral immunity affect CNS function (Hanamsagar and Bilbo 2017; Hammond, Robinton, and Stevens 2018; Zhao et al. 2018; Rothhammer et al. 2018) and mounting evidence that an array of

neurological disorders have a vascular and microglial component (Daneman 2012; Hammond, Robinton, and Stevens 2018; Zhao et al. 2018), a greater understanding of microglia-vascular crosstalk is necessary.

Juxtavascular microglial regulation of the vasculature

Evidence in the literature suggests a role for microglia in regulating vascular developmental. For example, in the embryonic brain, microglia are often localized to vascular junction points and mice depleted of microglia (PU.1 knockout mice) display decreases in vascular branching (Fantin et al. 2010). Similar findings have been reported in the developing retina (Rymo et al. 2011; Checchin et al. 2006; Yoshiaki Kubota et al. 2009; Dudiki et al. 2020). However, these studies are limited given that the genetic and pharmacological approaches used to deplete microglia target all monocyte-derived cells. My data demonstrating that microglia are localized to the vasculature prior to the arrival of astrocyte endfeet suggests juxtavascular microglia may be in a position to regulate vascular complexity and/or to help maintain the BBB prior to astrocyte endfeet arrival. Arguing against this, microglia depletion in the postnatal brain does not appear to induce changes in BBB integrity (Parkhurst et al. 2013; Elmore et al. 2014). Moreover, I demonstrate that mice with delayed microglial localization to the vasculature (*Cx3cr1*^{-/-} mice) do not have changes in vascular density during postnatal development. Additionally, I find that juxtavascular microglia preferentially associate with unsegmented vessels in the postnatal

brain, rather than branch points, providing further evidence that juxtavascular microglia may not regulate vascular complexity. Together, these data suggest that either peripheral myeloid cells can contribute to developmental vascular complexity or juxtavascular microglia may play differential roles in vascular development in embryonic versus postnatal development. Future experiments that specifically deplete microglia are required to identify the role microglia play in vascular development. Strategies include using a microglia-specific Cre line, such as *P2ry12* or *Hexb*, to deplete microglia embryonically and assess vascular complexity and BBB permeability in early postnatal and adult mice (McKinsey et al. 2020; Masuda et al. 2020).

Beyond regulating vascular development, microglia could play a role in regulating cerebral blood flow (CBF). The mammalian brain has evolved a unique mechanism for regional CBF control known as neurovascular coupling, where neuronal activity regulates CBF through communication with astrocytes, vascular smooth muscle cells, pericytes, and endothelial cells (Kisler et al. 2017). At the capillary level, where I find juxtavascular microglia predominantly localized, pericytes and astrocytes have been reported to play important roles in controlling blood flow. Previous work demonstrates that neuronal activity and the neurotransmitter glutamate evoke the release of messengers that dilate capillaries by actively relaxing pericytes (Hall et al. 2014). Additionally, astrocytes can mediate neurovascular signaling to capillary pericytes, influencing CBF (Mishra et al. 2016). Whether juxtavascular microglia could also influence CBF is

an open question. The close localization of juxtavascular microglia to astrocytes and pericytes at the vasculature suggests microglia could play similar roles. Additionally, microglia can respond to changes in neuronal activity (Dissing-Olesen et al. 2014; Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005; Tremblay, Lowery, and Majewska 2010; Schafer et al. 2012; Gunner et al. 2019), supporting a role for juxtavascular microglia in neurovascular coupling. Future experiments to address this open question include measuring the vascular diameter at juxtavascular microglia contact points before and after neuronal activity, such as whisker stimulation in the barrel cortex. Answers will impact our understanding of neurovascular coupling, which can become uncoupled in disease leading to vascular dysfunction and disease progression (Kisler et al. 2017; Iadecola 2017).

Vascular regulation of juxtavascular microglia

To date, the role of the vasculature in regulating microglia in the healthy brain has not been explored. My findings that microglia are intimately associated with the vasculature in both young and adult mice raises questions about the role the vasculature plays in the developmental changes that occur in microglia throughout the life of an animal (Hammond et al. 2019). Possible answers to these questions may be found in the periphery, where blood vessels can regulate macrophage differentiation and maturation of recruited monocytes, including proliferative status and phagocytic capacity (Krishnasamy et al. 2017).

Additionally, the differentiation of another glial cell type, OPCs, is regulated by the vasculature (Tsai et al. 2016). Whether the vasculature regulates juxtavascular microglia differentiation and maturation is unknown and represents an important future direction. In this thesis, I find that microglia are highly associated with the vascular basal lamina during the first week of postnatal development, as the microglial population is expanding and microglia are in a more immature, phagocytic state (Butovsky et al. 2014; Krasemann et al. 2017; Ashwell 1991; Perry, Hume, and Gordon 1985). These data point towards a possible role for the vasculature in regulating juxtavascular microglia, perhaps through modulating proliferative, phagocytic, or activation states of microglia. Interestingly, pericytes can serve as immune responsive cells which can enhance microglial activation (Matsumoto et al. 2014; 2018). This might imply a regulation by pericytes of microglial phenotype in the developing brain, however these experiments were performed *in vitro* and need to be validated *in vivo*. In the context of disease, the vasculature plays a role in regulating microglial activation (discussed in Part V of the discussion), which could provide insights into the healthy and developing brain. In the future, experiments aimed at addressing juxtavascular microglial states will be key to identify if the vasculature can regulate microglia. These include determining microglial proliferative and phagocytic state in juxtavascular versus vascular unassociated using markers such as Ki67 and CD68, respectively. Additionally, assessing juxtavascular microglia morphology could provide clues to vascular regulation of microglia, as

microglial morphology and function are closely related (Fernández-arjona et al. 2017). Finally, determining if juxtavascular microglia have a unique transcriptional profile compared to vascular-unassociated microglia could provide clues to if/how the vasculature regulates microglia. Methodologies to identify juxtavascular microglia specific genes will be discussed in Part IV of the discussion.

Another mechanism by which the vasculature could regulate microglia is through modulating juxtavascular microglia migration. As discussed in “*Possible mechanisms regulating juxtavascular microglia migration directionality and colonization*” in Part I of the discussion, vascular derived cues can modulate migration of newborn interneurons and neuroblasts. Here, I find that juxtavascular microglia are migratory on vessels during times of active colonization, raising the interesting hypothesis that vascular derived cues can regulate microglia migration and colonization. Identifying if the vasculature can regulate juxtavascular microglial migration will provide insight into microglial colonization as well as juxtavascular microglia-vascular crosstalk, furthering our understanding of juxtavascular microglia in the healthy brain.

Juxtavascular microglia as “first responders” to peripheral immune challenge

In the healthy brain, the CNS must be able to detect and respond to peripheral immune challenge and blood-born signals, but has no direct access to them (Persidsky et al. 2006; Saper 2010). Likewise, the immune system does not

directly contact the brain parenchyma, suggesting that they interact through a brain-immune interface. One intriguing possibility is that juxtavascular microglia serve as “first responders” to peripheral immune challenge. Here I demonstrate that juxtavascular microglia directly contact the vascular basal lamina, placing them in an ideal location to relay peripheral immune and blood-borne signals to the CNS.

A growing body of work has illustrated that peripheral immune activation can disrupt fetal brain development, and induce an onset of behavioral abnormalities in animal models (Hsiao et al. 2012; P. H. Patterson 2009). As the resident immune cells of the CNS and key regulators of neuronal development, microglia are thought to mediate a neuroinflammatory response that leads to neurodevelopmental disruptions. For example, bacterial infection in newborn rats alters the function of microglia such that a subsequent systemic lipopolysaccharide (LPS) injection results in exaggerated cytokine production within the brain, which is causally linked to cognitive deficits if the LPS infection occurs around the time of learning later in life (Williamson et al. 2011). Additionally, maternal immune activation (MIA) or LPS challenge in late adolescent mice has been shown to impact microglial transcriptional development, likely having significant consequences on neuronal development and increasing the risk for disease (Matcovitch-Natan et al. 2016; Hanamsagar et al. 2017). Environmental challenges to the immune system have also been shown to alter microglial phenotype. Exposure of pregnant dams to diesel

exhaust results in changes to microglial morphology in fetal brains, interestingly only in males, consistent with activation and/or delays in maturation in several brain regions (Bolton et al. 2017). Similarly, maternal exposure to ultrafine particles (UFP), a component of air pollution, leads to increases in corpus callosum size, hypermyelination, and microglial activation in offspring (Klocke et al. 2017). Although evidence is building that microglia communicate peripheral immune challenges to the CNS, the exact mechanism by which this occurs is unknown.

One intriguing site of communication between peripheral immune cells and microglia is the vasculature. Immune cells are found circulating in the blood and enter the brain through disruption of the BBB in disease (Engelhardt, Vajkoczy, and Weller 2017; Erickson and Banks 2018). In Chapter II, I demonstrate that juxtavascular microglia directly contact the vascular basal lamina in development and adulthood. As the basal lamina is a site of cell-cell communication (Obermeier, Daneman, and Ransohoff 2013), juxtavascular microglia could directly communicate with, or receive signals from, peripheral immune cells at these vascular contact sites. Alternatively, immune cells and juxtavascular microglia could communicate through a more indirect pathway. Under inflammatory conditions, endothelial cells and pericytes can serve as immune responsive cells, releasing cytokines and chemokines that can cause microglia activation (Hurtado-alvarado, Cabañas-morales, and Gómez-gonzález 2014; Xing et al. 2018; Navarro et al. 2016). Given the tight localization between

juxtavascular microglia, endothelial cells, and pericytes, it is possible that these cytokines and chemokines directly activate juxtavascular microglia, which could relay signals of peripheral immune challenge throughout the brain parenchyma. Determining if juxtavascular microglia act as “first responders” to immune challenges is important as early life immune activations, such as infection, toxin exposure, maternal stress, and metabolic disruptions, have increasingly been identified as a risk factor for several neurodevelopmental disorders such as ASDs, schizophrenia, and psychosis (P. Patterson 2011; A. S. Brown 2008; Boksa 2008; Dalman et al. 2008).

Concluding remarks

Since the first description of juxtavascular microglia in the early 1990's, there has been little research on these cells in the context of the healthy brain. Previous work suggests that juxtavascular microglia may play a role in vascular branching and complexity in the embryonic brain, however this work has several caveats and raises questions about juxtavascular microglia in the postnatal brain. In this thesis, I demonstrate that juxtavascular microglia are developmentally regulated, with a high percentage of microglia associated with the vasculature as they actively colonize the postnatal brain. Additionally, I find that microglia directly contact the basal lamina across all developmental time points, laying the groundwork to investigate microglia-vascular crosstalk in the healthy and developing brain. Future directions include gaining a deeper understanding of the

role juxtavascular microglia play in vascular development, as well as investigating if the vasculature can regulate microglia, a topic that has never been explored. In addition, as new evidence that microglia could be a conduit by which changes in peripheral immunity affect CNS function comes to light, it raises questions about the role of juxtavascular microglia as first responders to these immune challenges. Addressing these questions and gaining a deeper understanding of juxtavascular microglia-vascular cross talk in the healthy brain may provide insight into neurodegenerative diseases that are characterized by microglia activation, resulting in vascular dysfunction (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018).

Part IV. Juxtavascular microglia: a new subpopulation of microglia?

It has become increasingly appreciated that microglia are a heterogeneous population of cells that express different cellular markers, use different signaling mechanisms, and have differing morphologies across development and within different brain regions (Gunner et al. 2019; Masuda et al. 2019; Hammond et al. 2019; Tan, Yi, and Li 2020). Additionally, microglia that functionally differ from each other are thought to mix within close vicinity of the same anatomical site (De Biase and Bonci 2019; Hanisch 2013; Stratoulas et al. 2019), suggesting that microglia may differ within specific brain regions such that one subtype of microglia could be localized within multiple anatomical regions. This raises interesting questions about juxtavascular microglial identity. Could

juxtavascular microglia be a unique subpopulation of microglia localized throughout the brain? Future experiments aimed at identifying juxtavascular specific gene expression are required and could provide insight into their potential significance for normal CNS functions and in disease.

The vast majority of studies investigating microglia heterogeneity use a single-cell RNA-sequencing approach to characterize microglial gene expression from different regions of the brain that can easily be isolated, for example the cerebellum, cortex, hippocampus, and midbrain. Identifying if juxtavascular microglia are a unique population of cells by single-cell RNA-sequencing brings about technical difficulties since isolating juxtavascular microglia is not as simple as dissecting out different anatomical regions. One possible technique is to isolate brain vasculature and sequence juxtavascular cells that remain associated with the vessels. However, it has been reported that microglia do not stay bound to isolated vessels (Boulay et al. 2015), thus crosslinking reagents may be required to ensure juxtavascular microglia remain associated with the vessels, which could cause changes in gene expression. Alternatively, laser capture microdissection can be used to specifically isolate juxtavascular microglia, which can then be submitted for sequencing. While possible, laser capturing microglia can be challenging as these cells are small with little RNA, thus requiring capture of hundreds of cells (Mastroeni et al. 2017; 2018). The ideal method to identify if juxtavascular microglia are a unique population of cells is through spatial transcriptomics, such as multiplexed error-robust fluorescence

in situ hybridization (MERFISH) (K. H. Chen et al. 2015). Image-based approaches to single-cell transcriptomics naturally preserve the native spatial context of RNAs within a cell and the organization of cells within tissue (Moffitt et al. 2016). Moreover, new advances allow for the RNA profiling of hundreds of thousands of cells within 24 hours (Moffitt et al. 2016), making it an ideal technique to identify juxtavascular microglia specific gene expression.

Clues to genes specifically expressed in juxtavascular microglia can be taken from the literature and may provide insight into possible functions of these cells. In the embryonic brain, microglia are found highly associated with blood vessels and may perform proangiogenic functions (Fantin et al. 2010). In this context, vascular-associated microglia express TIE2 and neuropilin 1 (NRP1), two genes found to be significantly upregulated in proangiogenic tumor macrophages (Fantin et al. 2010; Pucci et al. 2009). However, it remains unknown if vascular-unassociated microglia also express these genes and if juxtavascular microglia continue expressing these genes throughout postnatal development and adulthood. Another interesting candidate molecule that may be specifically expressed in juxtavascular microglia is CD11c, a classic dendritic cell marker (Prodinger et al. 2011). Prodinger et al. investigated the distribution, phenotype, and source of CD11c⁺ cells in the non-diseased brain. Interestingly, they identified that the majority of CD11c⁺ cells were juxtavascular parenchymal cells that co-expressed ionized calcium-binding adaptor molecule 1 (IBA-1) and CD11b, markers also expressed in microglia (Prodinger et al. 2011). While

promising, they also identified a small population of CD11c+ cells within the perivascular space, suggesting these cells may not be microglia (Prodinger et al. 2011). In the future, identifying if these cells are microglia using microglia specific markers, such as P2RY12, will be important in determining if juxtavascular microglia are a new subpopulation of microglia.

In this thesis, I demonstrate that about 20% of microglia in the adult CNS are associated with the vasculature; raising the possibility that juxtavascular microglia may be a new microglial subpopulation. Investigation into microglial heterogeneity has demonstrated that microglia function, phenotype, and gene expression can vary across brain regions. Considering the importance of microglia for CNS development and homeostasis, this regional heterogeneity suggests microglia may selectively influence CNS functions and contribute to neurological diseases. As juxtavascular microglia contribute to vascular dysfunction in the diseased CNS (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018), understanding if these cells are a new subpopulation of microglia could provide therapeutic strategies to specifically target juxtavascular microglia and ameliorate vascular dysfunction.

Part V. Juxtavascular microglia: implications for CNS disease

The data presented in this thesis have important implications for neurological diseases associated with the injured or aged CNS, such as Multiple Sclerosis (MS), Alzheimer's disease (AD), stroke, and peripheral inflammation,

where there is enhanced microglia-vascular association and vascular dysfunction, as outlined in the introduction “*Juxtavascular microglia in the diseased CNS*”. In Chapter II, I demonstrate that juxtavascular microglia directly contact the vascular basal lamina in areas devoid of astrocyte endfeet, raising the possibility that these contact sites may be more vulnerable to BBB breakdown and peripheral immune cell invasion. Indeed, under inflammatory conditions, endothelial cells and pericytes can serve as immune responsive cells, releasing cytokines and chemokines that can cause microglia activation (Hurtado-alvarado, Cabañas-morales, and Gómez-gonzález 2014; Xing et al. 2018; Navarro et al. 2016). When activated, microglia secrete a range of toxic molecules such as ROS, NO, PGE, COX-2, quinolinic acid, several chemokines such as MCP-1, CXCL-1, and MIP-1 α , and pro-inflammatory cytokines such as IL-6, TNF- α , and IL-1 β , all of which exert a detrimental effect on the integrity and function of the BBB (Morris et al. 2018; Sousa et al. 2018). Given the tight localization between juxtavascular microglia, endothelial cells, and pericytes, it is possible that endothelial cells and pericytes can directly activate juxtavascular microglia, leading to BBB breakdown. Alternatively, these sites of contact may strengthen the BBB and initially prevent its breakdown. Recent work demonstrates that during systemic inflammation, vessel-associated microglia initially maintain BBB integrity via expression of the tight-junction protein Claudin-5 and make physical contact with the endothelial cells (Haruwaka et al. 2019). However, during sustained inflammation, microglia phagocytose astrocytic end-

feet and impair BBB function (Haruwaka et al. 2019). Together, these data suggest that juxtavascular microglia localization to the vasculature in areas devoid of astrocyte endfeet may play a dual role in maintaining BBB integrity in the diseased brain.

In models of stroke, microglia become juxtavascular 24 hours after ischemic insult (Jolivel et al. 2015). Interestingly, mice deficient in CX3CR1, displayed a reduction in the size of the ischemic infarct area and BBB damage (Dénes et al. 2008; Jolivel et al. 2015; Z. Tang et al. 2014). However, how CX3CR1 reduces ischemic and BBB damage is an open question. In this thesis, I demonstrate that CX3CR1 regulates the timing of microglia-vascular interactions, raising the intriguing hypothesis that *Cx3cr1*^{-/-} microglia are delayed in their localization to the vasculature under stroke conditions resulting in reduced BBB damage. Supporting this, a selective inactivation of microglial CX3CR1 that has been reported to regulate microglial migration (Liang et al. 2009; A. E. Cardona et al. 2006), significantly reduced blood extravasation (Jolivel et al. 2015), pointing towards a role for CX3CR1-dependent timing of juxtavascular microglia in BBB damage and immune cell extravasation after stroke. As CX3CR1 also plays a role in the progression of other neurological diseases characterized by vascular dysfunction, such as AD (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018; Finneran and Nash 2019), understanding if CX3CR1-dependent timing of microglia-vascular interactions contributes to disease could provide novel therapeutic strategies.

In addition to diseases of the aged and injured CNS, data presented in this thesis have implications for neurodevelopmental disorders such as ASDs. Microglial colonization of the appropriate brain regions at the correct time during development is critical for proper neuronal circuit development (Paolicelli et al. 2011; Hoshiko et al. 2012; Zhan et al. 2014). Mice with delayed colonization of synapse-dense brain regions display long-lasting behavior deficits associated with ASDs (Zhan et al. 2014). Moreover, in human ASDs patients, disruptions in microglial density and synaptic connectivity during critical periods of development have been identified (Edmonson, Ziats, and Rennert 2016; Morgan et al. 2010). Thus, understanding how microglia colonize the brain at the correct time will impact our understanding of ASDs. In Chapter II, I demonstrate that juxtavascular microglia migrate on blood vessels, which may be important for the timing of microglial colonization to synapse-dense brain regions where they regulate synapse maturation and pruning during critical windows in development (Paolicelli et al. 2011; Tremblay, Lowery, and Majewska 2010; Schafer et al. 2012; Gunner et al. 2019; Hoshiko et al. 2012). If these interactions are disrupted, the timing of synapse development and, ultimately, neural circuit function may be altered. This is supported by my data that *Cx3cr1*^{-/-} mice show delays in microglial association with the vessels, which is concomitant with known delays in microglial recruitment to developing synapses and delays in synapse maturation in these mice (Paolicelli et al. 2011; Zhan et al. 2014; Hoshiko et al. 2012). Future studies investigating microglia-vascular interactions

in the ASDs human brain will be necessary to determine the importance of juxtavascular microglia in neurodevelopmental disorders.

Together, my thesis work investigating juxtavascular microglia in the healthy brain provides insights into CNS diseases characterized by microglia and vascular dysfunction, as well as aberrant microglial colonization. Understanding precisely when, where, and mechanisms by which microglia interact with the vasculature in the healthy brain may lead to therapeutic strategies to reduce vascular pathology and facilitate recovery.

Part VI. Concluding remarks

The work presented in this thesis sheds new light on an understudied population of microglia, juxtavascular microglia. I provide the first extensive characterization of microglia-vascular interactions in the healthy, postnatal brain and identify a molecular mechanism regulating the timing of these interactions during development. I determine that juxtavascular microglia are developmentally regulated, with a high percentage of microglia associated with and migratory on blood vessels, concomitant with a peak in microglial colonization of the cortex and recruitment to synapses (Fig 4.1A). Juxtavascular microglia become stationary on vessels as astrocyte endfeet arrive and the brain matures. 2-photon live imaging in adult mice reveals that these vascular-associated microglia in the mature brain are stable and stationary for several weeks (Fig 4.1A). Finally, I provide evidence that the timing of these interactions during development is

regulated by CX3CR1, but not CX3CL1, the canonical *in vivo* ligand for CX3CR1, and hypothesize that this delay in microglia-vascular interactions leads to delays in microglial colonization of the barrel cortex in CX3CR1 deficient mice (Fig 4.1B). Together, these data lay the fundamental groundwork to investigate the role of the vasculature in microglial colonization, juxtavascular microglia-astrocyte crosstalk, juxtavascular microglia function, and juxtavascular microglia as a novel subpopulation of microglia in the healthy and diseased brain. Moreover, data presented in this thesis have important implications for neurological diseases associated with the injured or aged CNS, such as Multiple Sclerosis, Alzheimer's disease, stroke, and peripheral inflammation, where there is enhanced microglia-vascular association and vascular dysfunction.

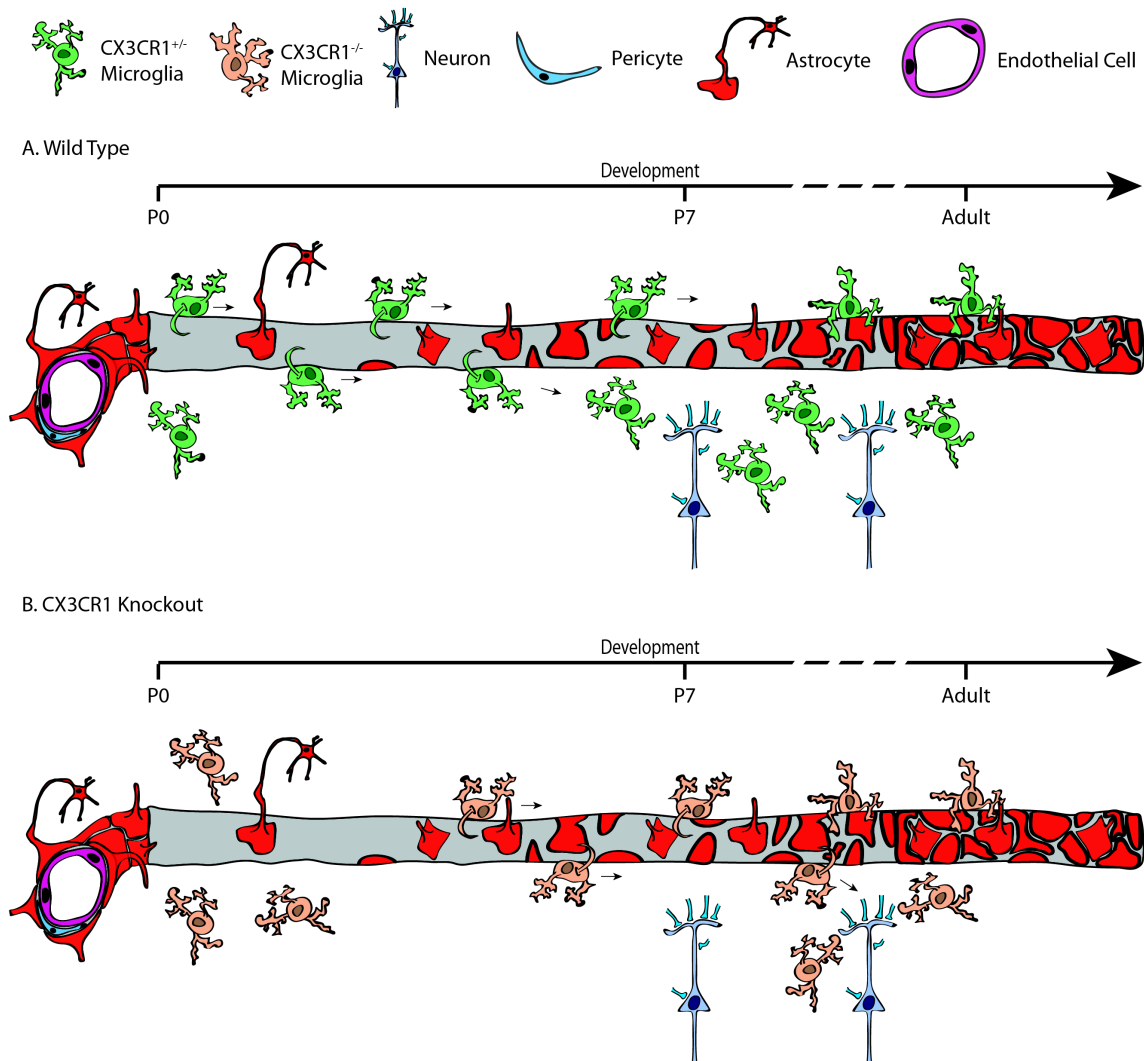


Figure 4.1: Working model of microglia-vascular interactions in wild type and CX3CR1 deficient mice. A. During the first week of postnatal development in wild type animals, a high percentage of microglia are juxtavascular, coinciding with a peak in microglial colonization of the cortex and recruitment to synapses. These juxtavascular microglia are motile during times of active microglial colonization, but become stationary and stable on blood vessels long-term in adulthood. This change in microglial association and dynamics is concomitant with expansion of astrocyte endfoot territory on the vasculature. This data supports a mechanism by which microglia use the vasculature to migrate and colonize the developing brain parenchyma. This migration becomes restricted upon the arrival of astrocyte endfeet such that juxtavascular microglia become highly stationary and stable in the mature cortex. **B.** CX3CR1 deficient mice have delays in microglia-vascular interactions that coincide with delays in microglial colonization of the synapse dense barrel cortex. These data are consistent with a

model in which microglia associate with the vasculature in a CX3CR1-dependent manner and use the vasculature to localize to the appropriate brain regions at the correct time. Inefficient microglial association with the vasculature in CX3CR1 deficient mice may lead to disruptions to the timing of juxtavascular microglia and subsequent migration and colonization, which is resolved in adult animals where juxtavascular microglia and microglial colonization reach wild type levels.

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