

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

**Running Title: Developmental analysis of juxtavascular microglia**

Authors: Erica Mondo<sup>1</sup>, Shannon C. Becker<sup>1#</sup>, Amanda G. Kautzman<sup>1#</sup>, Martina Schifferer<sup>2</sup>, Christina E. Baer<sup>3</sup>, Jiapei Chen<sup>4,5</sup>, Eric J. Huang<sup>4,5</sup>, Mikael Simons<sup>2,6,7</sup>, and Dorothy P. Schafer<sup>1\*</sup>

Affiliations:

<sup>1</sup>Department of Neurobiology, University of Massachusetts Medical School, Worcester, MA 01605, USA

<sup>2</sup>German Center for Neurodegenerative Disease, Munich, Germany

<sup>3</sup>Sanderson Center for Optical Experimentation, University of Massachusetts Medical School, Worcester, MA 01605, USA

<sup>4</sup>Department of Pathology, University of California, San Francisco, San Francisco, CA 94143, USA

<sup>5</sup>Pathology Service (113B), San Francisco VA Medical Center, San Francisco, CA 94121, USA.

<sup>6</sup>Institute of Neuronal Cell Biology, Technical University Munich, Munich, Germany

<sup>7</sup>Munich Cluster of Systems Neurology (SyNergy), Munich, Germany

#Authors contributed equally

\*Corresponding Author:

Dorothy Schafer

[Dorothy.schafer@umassmed.edu](mailto:Dorothy.schafer@umassmed.edu)

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1 **ABSTRACT**

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

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27 **SIGNIFICANCE STATEMENT**

28 We report the first extensive analysis of juxtavascular microglia in the healthy, developing and  
29 adult brain. Live imaging revealed that juxtavascular microglia within the cortex are highly motile  
30 and migrate along vessels as they are colonizing cortical regions. Using confocal, expansion,  
31 super-resolution, and electron microscopy, we determined that microglia associate with the  
32 vasculature at all ages in areas lacking full coverage astrocyte endfoot coverage and motility of  
33 juxtavascular microglia ceases as astrocyte endfeet more fully ensheath the vasculature. Our  
34 data lay the fundamental groundwork to investigate microglia-astrocyte crosstalk and  
35 juxtavascular microglial function in the healthy and diseased brain. They further provide a  
36 potential vascular-dependent mechanism by which microglia colonize the brain to later regulate  
37 neural circuit development.

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## 53 INTRODUCTION

54 While myeloid lineage in origin, microglia are now appreciated to be key cellular  
55 components of neural circuits. Imaging studies have revealed that microglia are constantly  
56 extending and retracting their processes, which are in frequent contact with neurons, synapses,  
57 and other glial cells (Davalos et al. 2005; Nimmerjahn, Kirchhoff, and Helmchen 2005; Schafer  
58 et al. 2012; Tremblay, Lowery, and Majewska 2010; Frost and Schafer 2016). These  
59 descriptions of physical interactions between microglia and other resident CNS cell types have  
60 now led to a new understanding that microglia are important for neural circuit structure and  
61 function, including their role in developmental synaptic pruning by engulfing and removing  
62 synapses from less active neurons (Schafer et al. 2012; Tremblay, Lowery, and Majewska  
63 2010; Paolicelli et al. 2011; Gunner et al. 2019). Besides interactions with parenchymal neurons  
64 and glia, microglia are known to interact with the vasculature. However, the vast majority of  
65 these studies have been in the context of disease where parenchymal microglia rapidly  
66 associate with the brain vasculature following breakdown of the blood-brain barrier (BBB) and,  
67 in turn, inflammatory microglia can modulate the breakdown of the BBB (Stankovic, Teodorczyk,  
68 and Ploen 2016; Zhao et al. 2018). Far less is known about how microglia interact with the  
69 vasculature in the healthy brain. With new evidence that microglia could be a conduit by which  
70 changes in peripheral immunity (e.g. microbiome, infection, etc.) affect CNS function  
71 (Hanamsagar and Bilbo 2017; Hammond, Robinton, and Stevens 2018; Zhao et al. 2018;  
72 Rothhammer et al. 2018) and mounting evidence that an array of neurological disorders have a  
73 vascular and microglial component (Daneman 2012; Hammond, Robinton, and Stevens 2018;  
74 Zhao et al. 2018), a greater understanding of microglia-vascular interactions is necessary.

75 The neurovascular unit (NVU) is composed of endothelial cells, pericytes, vascular  
76 smooth muscle cells, astrocytes, macrophages, and neurons that connect the brain parenchyma  
77 to the cerebral vasculature. Interactions between these NVU cell types is important for a variety  
78 of physiological processes such as angiogenesis, vessel maintenance and permeability,

79 metabolic support, and regulation of blood flow (Brown et al. 2019; McConnell et al. 2017). The  
80 development of the NVU begins around embryonic day (E) 9.5 in mice, when specialized  
81 endothelial cells branch from vessels of the perineural vascular plexus to form capillaries that  
82 invade nearby neural tissue (Saili et al. 2017). Pericytes associate with endothelial cells as  
83 nascent vessels generate at E9.5 (Armulik et al. 2010; Bauer et al. 1993; Yamanishi et al. 2012;  
84 Daneman et al. 2010) and these interactions are critical to form the BBB (Zlokovic 2008;  
85 Daneman et al. 2010). Astrocytes are also a key component of the mature NVU. After the  
86 vasculature initially forms, astrocytes extend their processes to form endfeet over the course of  
87 postnatal development in rodents (Daneman et al. 2010). These astrocyte endfeet ultimately  
88 surround and ensheath the majority of the vasculature by adulthood where they play roles in a  
89 variety of functions such as maintaining the BBB, providing metabolic support to neurons, and  
90 regulating blood flow (Abbott, Rönnbäck, and Hansson 2006; Kimelberg and Nedergaard 2010;  
91 Macvicar and Newman 2015).

92         The vast majority of studies assessing interactions between microglia and the  
93 vasculature are in the context of disease. For example, microglia rapidly surround and contact  
94 the vasculature following breakdown of the BBB in the inflamed CNS (Zhao et al. 2018;  
95 Stankovic, Teodorczyk, and Ploen 2016). One mechanism regulating these microglia-vascular  
96 interactions is the blood component fibrinogen and CD11b on microglia (Davalos et al. 2012;  
97 Adams et al. 2007). Reactive microglia can also influence the opening of the BBB by  
98 phagocytosing astrocyte endfeet or upregulating molecules such as VEGF, iNOS, and ROS  
99 (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al. 2018; Haruwaka et al. 2019). In the  
100 healthy brain, much less is known. Studies in rodents and humans have shown that microglia  
101 associate with the vasculature in the developing CNS and live imaging in postnatal brain slices  
102 following traumatic injury or in embryonic mouse brain slices has suggested that microglia can  
103 migrate along the vasculature (Monier et al. 2007; Fantin et al. 2010; Smolders et al. 2017;  
104 Grossmann et al. 2002; Checchin et al. 2006). Microglia have also been suggested to regulate

105 vascular growth and complexity in the developing hindbrain and retina (Fantin et al. 2010; Rymo  
106 et al. 2011; Checchin et al. 2006; Yoshiaki Kubota et al. 2009; Dudiki et al. 2020). Together,  
107 these studies provide evidence that there is microglia-vascular crosstalk, which requires further  
108 investigation in development, adulthood, and disease.

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

119

## 120 **MATERIALS AND METHODS**

### 121 **Animals**

122 Male and female mice were used for all experiments. *Cx3cr1*<sup>-/-</sup> mice (*Cx3cr1*<sup>EGFP/EGFP</sup>; stock  
123 #005582) and *C57Bl/6/J* (stock #000664) mice were obtained from Jackson Laboratories (Bar  
124 Harbor, ME). Heterozygous breeder pairs were set up for all experiments and wild-type (WT)  
125 and heterozygote littermates were used as controls with equal representation of males and  
126 females for each genotype. All experiments were performed in accordance with animal care and  
127 use committees and under NIH guidelines for proper animal welfare.

128

### 129 **Human prenatal brain collection and immunofluorescence microscopy**

130 Deidentified prenatal human brain tissues were collected via the Department of Pathology  
131 Autopsy Service at the University of California San Francisco under the approval of the  
132 Committee on Human Research (CHR, Study #: 12-08643). Brain tissues from four prenatal  
133 cases at 15, 18, 21 and 28 gestational weeks (GW) were evaluated using standard  
134 neuropathologic examinations to rule out any gross or microscopic abnormalities. These  
135 autopsy cases, which all had postmortem intervals of less than 48 hours, were fixed in freshly  
136 prepared 4% paraformaldehyde (PFA) and sampled at the level of the mammillary body.  
137 Following fixation in 4% PFA for 48 hours, brain samples were incubated with 20% sucrose  
138 solution, and were frozen in embedding medium OCT for cryosectioning at 20 $\mu$ m. For  
139 consistency, 3-6 consecutive sections were prepared from each sample and immunostained  
140 with anti-Iba1 antibody (Wako; Richmond, VA; 1:3000) and anti-CD31 antibody (R&D Systems;  
141 Minneapolis, MN; 1:200). Images of the ventricular and subventricular zones at the level of the  
142 frontal cortex were acquired on Leica SP8 confocal microscope using a 40X (1.3NA) objective  
143 lens.

144

#### 145 **Preparation of tissue for immunofluorescence microscopy**

146 Mice were perfused with 1X Hank's balanced salt solution (HBSS) -magnesium, -calcium,  
147 (Gibco, Gaithersburg, MD) prior to brain removal at indicated ages. For analysis of frontal and  
148 somatosensory cortex, brains were post-fixed in 4% paraformaldehyde in 0.1M phosphate  
149 buffer (PB) for four hours. Brains were placed in 30% sucrose in 0.1M PB and allowed to sink  
150 prior to sectioning. Sections were blocked in 10% goat serum, 0.01% TritonX-100 in 0.1M PB  
151 for 1 hour before primary immunostaining antibodies were applied overnight. Secondary  
152 antibodies were applied for two hours the following day. All steps were carried out at room  
153 temperature with agitation. For structured illumination microscopy (SIM), sections were blocked  
154 in 3% BSA, 0.01% TritonX-100 in 0.1M PB for 1 hour before primary immunostaining antibodies  
155 were applied for 48 hours at 4°C. Secondary antibodies were applied for four hours at room

156 temperature with agitation. The following antibodies were used: anti-P2RY12 (Butovsky  
157 Laboratory, Brigham and Women's Hospital, Harvard University; 1:200), anti-PECAM  
158 (Biolegend; San Diego, CA; 1:100), anti-aquaporin 4 (Millipore Sigma; St. Louis, Missouri;  
159 1:200), anti-Pdgfr $\beta$  (Thermo Fisher Scientific; Waltham, MA; 1:200), anti-Lyve1 (Abcam;  
160 Cambridge, MA; 1:200), anti-smooth muscle actin (SMA) (Millipore Sigma; St. Louis, Missouri;  
161 1:200) and anti-VGLuT2 (Millipore Sigma; St. Louis, Missouri; 1:2000).

162

### 163 **Confocal microscopy**

164 Immunostained sections were imaged on a Zeiss Observer Spinning Disk Confocal microscope  
165 equipped with diode lasers (405nm, 488nm, 594nm, 647nm) and Zen acquisition software  
166 (Zeiss; Oberkochen, Germany). For microglia-vascular interaction, microglial density, microglia  
167 association with SMA+ or SMA- vessels and vascular density analyses, 20x, single optical  
168 plane, tiled images of the frontal or somatosensory cortex were acquired for each animal. To  
169 create a field of view (FOV), each tiled image was stitched using Zen acquisition software. Two  
170 FOVs (ie. tiled images) were acquired per animal. To note, anti-P2RY12 immunostaining was  
171 used to label microglia in wild type animals, which was more difficult to visualize at lower  
172 magnification at older ages compared to EGFP-labeled microglia. As a result, for anti-P2RY12  
173 immunostained sections from P7-P28 animals, twelve 40x fields of view were acquired per  
174 animal with 76 z-stack steps at 0.22 $\mu$ m spacing. For analysis of vascular diameter,  
175 juxtavascular association with branched/unsegmented vessels, primary processes aligned with  
176 vessels, astrocyte endfeet coverage on the vasculature, and vascular-associated microglia  
177 contacts with astrocytes, six-twelve 40X fields of view were acquired from the frontal cortex per  
178 animal with 76 z-stacks at 0.22 $\mu$ m spacing.

179

### 180 **Juxtavascular microglia and microglia density analyses in the frontal and somatosensory** 181 **cortices**

182 Using the DAPI channel as a guide, a region of interest (ROI) was chosen in each cortical layer,  
183 I-VI from each 20x stitched tiled image (10 ROIs per animal). Subsequent images were  
184 analyzed in ImageJ (NIH; Bethesda, MD). For anti-P2RY12, sections were acquired at 40x, a  
185 maximum intensity projection was made from each z-stack and was considered a ROI (12 per  
186 animal). The ROI areas were recorded. The same ROI was transposed on the microglial  
187 channel and the cell counter ImageJ plugin was used to count the number of microglia in the  
188 ROI. The total density of microglia was then calculated by dividing the microglia number by the  
189 ROI area. To assess microglial association with the vasculature, the microglia and blood vessel  
190 channels were merged and the cell counter plugin was used to manually count the number of  
191 microglia with cell bodies contacting blood vessels. Juxtavascular microglia were defined as  
192 microglia with at least 30% of their soma perimeter in contact with blood vessels and soma  
193 centers that were within 10 $\mu$ m of the vessel. The percent of juxtavascular microglia was  
194 calculated by summing the total number of microglia on vasculature divided by the total number  
195 of microglia within the ROI. For each animal, data from the ROIs were averaged together to get  
196 a single average per animal for statistical analyses.

197

### 198 **Juxtavascular microglia analysis within the barrel cortex**

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

208 leaving behind only the septa fluorescence and the cell counter plugin was again used to count  
209 the number of microglia and the number of juxtavascular microglia in the septa. To calculate the  
210 percent of juxtavascular microglia in the barrel cortex, the total numbers of juxtavascular  
211 microglia in the barrels and septa were summed and divided by the total number of microglia in  
212 the barrel and septa, respectively, for each ROI. The total microglia in barrels and septa,  
213 regardless of vascular association, were also calculated. All numbers across 12-18 cropped  
214 images were then averaged for a given animal prior to statistical analyses.

215

### 216 **Vascular density analysis**

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

223

### 224 **Microglial association with SMA+ or SMA- vessels analysis**

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

234

### 235 **Vascular diameter analysis**

236 Using Imaris (Bitplane) software, the diameter of the vessel was measured in 3D at microglial  
237 soma contact points from 40X images (12 per animal). For each animal, data from the 12  
238 images were averaged together to get a single average per animal for statistical analysis.

239

### 240 **Primary Process and branched/unsegmented vessel analyses**

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

251

### 252 **Acute Slice Time-Lapse Imaging**

253 Mice were given a retro-orbital injection of Texas Red labeled dextran (Fisher Scientific;  
254 Waltham, MA) 10 minutes prior to sacrifice to label vasculature. Mice were euthanized at P7 or  
255 P $\geq$ 120, brains were isolated and sectioned coronally at a thickness of 300 $\mu$ m using a Leica  
256 VT1200 vibratome in oxygenated 37°C artificial cerebrospinal fluid (ACSF). Slices were  
257 mounted on a MatTak glass bottom microwell dish and placed in a Zeiss Observer Spinning  
258 Disk Confocal microscope equipped with diode lasers (405nm, 488nm, 594nm, 647nm) and Zen  
259 acquisition software (Zeiss; Oberkochen, Germany). Image acquisition started after a minimum



260 of 30 minutes of tissue equilibration at 37°C with 5% CO<sub>2</sub> and within 2 hours of decapitation.  
261 Oxygenated ACSF was continuously perfused over the slices at a rate of 1.5-2µm/minute for the  
262 duration of equilibration and imaging. Per animal, one field of view was imaged every 5 minutes  
263 over 6 hours on an inverted Zeiss Observer Spinning Disk Confocal and a 20X objective. Z-  
264 stacks spanning 50-60µm, with serial optical sections of 1.5-2µm were recorded from a minimal  
265 depth of 30µm beneath the surface of the slice to avoid cells activated by slicing.

266

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

268 Cranial window surgeries were performed as previously described within the visual cortex  
269 (2.5µm lateral and 2.0 µm posterior from bregma) (Goldey et al. 2014). One week after surgery,  
270 mice were head-fixed to a custom-built running wheel and trained to run while head restrained  
271 for increasing time intervals several days a week. Two weeks post surgery long-term 2-photon  
272 live imaging began. Mice were given a retro-orbital injection of Texas Red labeled dextran  
273 (Fisher Scientific; Waltham, MA) 10 minutes prior to imaging and were head restrained on a  
274 custom built running wheel, which was positioned directly under the microscope objective.  
275 Images were acquired with a 20X water immersion objective (Zeiss, NA 1.0) on a Zeiss Laser  
276 Scanning 7 MP microscope equipped with a tunable coherent Chameleon Ultra II multiphoton  
277 laser and BiG detector. Three different regions of interest (ROIs) were taken at least 75µm  
278 below the surface of the brain, with z-stacks spanning 45-65µm with a step size of 2.5µm for  
279 each animal. On the first day of imaging, each ROI was imaged every 5 minutes over 2 hours.  
280 The same ROIs were then imaged once (single z-stack) on the following days post first imaging  
281 session: 1, 3, 7, 10, 14, 17, 21, 24, 28, 35, and 42 days. For each imaging day, the ROIs from  
282 day 0 of imaging were identified based on the vascular structure.

283

### 284 **Migration tracking and analysis**

285 Image processing and microglial soma motility/migration tracking were performed using ImageJ  
286 (NIH; Bethesda, MD). Time series were first corrected for 3D drift using the 3D drift correction  
287 plugin (Parslow, Cardona, and Bryson-richardson 2014) and migration was tracked using the  
288 TrackMate plugin (Tinevez et al. 2017). For each developmental time point, 10-12 juxtavascular  
289 and vascular-unassociated microglia were analyzed per animal (n=4 mice per developmental  
290 time point). Only cells remaining in the field of view for six hours were included in the analysis.  
291 The average soma motility ( $\mu\text{m}/\text{h}$ ) was calculated by measuring the displaced distance of the  
292 microglial soma between time=0 min and time=360 min and dividing by the duration of the  
293 imaging session. Juxtavascular distance migrated was calculated by measuring the displaced  
294 distance of the microglial soma between time=0 min and time=360 min. Juxtavascular migration  
295 trajectory was calculated by measuring the angle between the blood vessel and juxtavascular  
296 microglia soma along the longest, continuous stretch of motility on the vessel. Percent of cells  
297 within each binned category (motility, distance travelled, and trajectory) was calculated by  
298 dividing the number microglia of within each category by the total number of microglia. For each  
299 animal, data from each analyzed cell were averaged together to get a single average per animal  
300 for statistical analysis.

301 *In vivo* tracking of juxtavascular microglia motility and long-term juxtavascular microglia  
302 were performed using ImageJ (NIH; Bethesda, MD). Time series were first corrected for 3D drift  
303 using the 3D drift correction plugin (Parslow, Cardona, and Bryson-richardson 2014) and  
304 migration was tracked using the TrackMate plugin (Tinevez et al. 2017). To calculate percent of  
305 microglia stationary over two hours, the number of stationary juxtavascular and vascular-  
306 unassociated microglia was divided by the total number of microglia. To calculate the percent of  
307 original juxtavascular microglia that remain on vessels over 42 days, the number of  
308 juxtavascular microglia on day 0 was calculated. For each subsequent day, the number of these  
309 original juxtavascular microglia that were still associated with the vasculature was determined  
310 and divided by the number of original juxtavascular microglia on day 0. For each animal, data

311 was analyzed from three ROIs and averaged together to get a single average per animal for  
312 statistical analysis. For each animal, data was analyzed from three ROIs and added together to  
313 get a single average per animal for statistical analysis.

314

#### 315 **Astrocyte endfeet coverage analysis**

316 Using Imaris (Bitplane) software, the astrocyte endfeet and vessel channels were 3D rendered  
317 from 40X images (6 per animal). The astrocyte channel was then masked onto the vessel  
318 channel and the masked astrocyte channel was 3D rendered. Volumes of the 3D rendered  
319 vessel channel and masked astrocyte endfeet channel were recorded. The percent of blood  
320 vessels covered by astrocyte endfeet was calculated by dividing the blood vessel volume by the  
321 masked astrocyte endfeet volume. For each animal, data from the 6 images were averaged  
322 together to get a single average per animal for statistical analysis.

323

#### 324 **Juxtavascular microglia- astrocyte contact**

325 Analysis was done using the same images used for astrocyte endfeet coverage analysis in  
326 Imaris (bitplane). The microglia was 3D rendered, masked onto the blood vessel and astrocyte  
327 endfeet channel, and the volume of the masked microglial channel was recorded. The percent  
328 of juxtavascular microglia contacting blood vessels only, vessels and astrocyte endfeet, or  
329 astrocyte endfeet only was calculated by summing the number of microglia contacting vessels  
330 only, vessels and astrocyte endfeet, or astrocyte endfeet only and dividing by the total number  
331 of juxtavascular microglia. For each animal, data from the 6 images were averaged together to  
332 get a single average per animal for statistical analysis.

333

#### 334 **Expansion Microscopy (ExM)**

335 Expansion microscopy was performed as previously described (Asano et al. 2018) with slight  
336 modification. Briefly, 80µm floating sections were blocked in 0.5% bovine serum albumin (BSA)

337 and 0.3% Triton-X100 (TX-100) for 1 hour at room temperature. Primary antibodies, anti-  
338 aquaporin 4 (Millipore Sigma; St. Louis, Missouri; 1:200), anti-PDGFR $\beta$  (Thermo Fisher  
339 Scientific; Waltham, MA; 1:100), and anti-GFP (Abcam; Cambridge, MA; 1:200) were incubated  
340 in 0.5% BSA and 0.3% TX-100 at 4°C for 4 nights. Secondary antibodies were added at 1:200  
341 dilutions overnight at room temperature. Expansion microscopy protocol (Basic Protocol 2) was  
342 then followed as published in Asano et al. 2018.

343

#### 344 **Structured Illumination Microscopy (SIM)**

345 Structured Illumination Microscopy (SIM) was performed using a GE Delta Vision OMX V4  
346 microscope with pCO.edge sCMOS cameras and an Olympus 60x 1.42 NA objective. Samples  
347 were mounted in Prolong Glass mounting media with #1.5 coverslips and imaged using 1.516  
348 refractive index immersion oil. Image processing was completed using the GE softWorx  
349 software and image quality was determined using the SIMcheck plugin in ImageJ. SIM figures  
350 were produced in ImageJ (NIH; Bethesda, MD).

351

#### 352 **Scanning Electron Microscopy (SEM)**

353 Mice were perfusion fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium  
354 cacodylate buffer at pH 7.4 (Science Services). Brains were dissected, vibratome sectioned,  
355 and immersion fixed for 24h at 4°C. We applied a rOTO (reduced osmium-thiocarbohydrazide-  
356 somium) staining procedure adopted from Tapia et al. (Tapia et al. 2013). Briefly, the tissue was  
357 washed and post-fixed in 2% osmium tetroxide (EMS), 2% potassium hexacyanoferrate (Sigma)  
358 in 0.1 M sodium cacodylate buffer. After washes in buffer and water the staining was enhanced  
359 by reaction with 1% thiocarbohydrazide (Sigma) for 45 min at 50°C. The tissue was washed in  
360 water and incubated in 2% aqueous osmium tetroxide. All osmium incubation steps were carried  
361 out over 90 min with substitution by fresh reagents after 45 min, respectively. To further intensify  
362 the staining, 2% aqueous uranyl acetate was applied overnight at 4°C and subsequently

363 warmed to 50°C for 2h. The samples were dehydrated in an ascending ethanol series and  
364 infiltrated with LX112 (LADD). The samples were flat embedded into gelatin capsules (Science  
365 Services) and cured for 48h. The block was trimmed by 200 µm at a 90° angle on each side  
366 using a TRIM90 diamond knife (Diatome) on an ATUMtome (Powertome, RMC). Consecutive  
367 sections were taken using a 35° ultra-diamond knife (Diatome) at a nominal cutting thickness of  
368 100 nm and collected on freshly plasma-treated (custom-built, based on Pelco easiGlow,  
369 adopted from Mark Terasaki) CNT tape (Yoshiyuki Kubota et al. 2018). We collected 450 (P5)  
370 and 550 (P56) cortical sections, covering a thickness of 45-55 µm in depth. Tape strips were  
371 mounted with adhesive carbon tape (Science Services) onto 4-inch silicon wafers (Siebert  
372 Wafer) and grounded by additional adhesive carbon tape strips (Science Services). EM  
373 micrographs were acquired on a Crossbeam Gemini 340 SEM (Zeiss) with a four-quadrant  
374 backscatter detector at 8 kV. In ATLAS5 Array Tomography (Fibics), the whole wafer area was  
375 scanned at 3000 nm/pixel to generate an overview map. The entire ultrathin section areas of  
376 one wafer (314 sections (P5), 279 sections (P56)) were scanned at 100 x 100 x 100 nm<sup>3</sup> (465 x  
377 638 µm<sup>2</sup> (P5), 1249 x 707 µm<sup>2</sup> (P56). After alignment in Fiji TrakEM2 (Cardona et al. 2012)  
378 areas that contained microglia in close proximity to blood vessels (148 x 136 x 16 µm<sup>3</sup> (P5), 193  
379 x 186 x 12 µm<sup>3</sup> (P56) were selected for high resolution acquisition. We collected 29 total 2D  
380 micrographs (10 x 10 nm<sup>2</sup>) from n=3 animals at P5 and 11 total micrographs from n=3 animals  
381 at P56. From each age, one juxtavascular microglia was identified and selected to generate a  
382 3D volume (10 x 10 x 100 nm<sup>3</sup>). The image series were aligned in TrakEM2 using a series of  
383 automated and manual processing steps. For the P5 and P56 image series, segmentation and  
384 rendering was performed in VAST (Volume And Segmentation Tool) (Berger et al. 2018). We  
385 used Blender to render the two 3D models (Community 2018).

386

387 **Statistical analyses**

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

393

## 394 **RESULTS**

### 395 **A high percentage of microglia are juxtavascular during development**

396 During rodent and human embryonic development, microglia somas have been  
397 described to be in close contact with blood vessels (Fantin et al. 2010; Monier et al. 2007;  
398 Checchin et al. 2006). We assessed microglial association with the vasculature over an  
399 extended developmental time course across postnatal development. Microglia were labeled  
400 using transgenic mice that express EGFP under the control of the fractalkine receptor CX3CR1  
401 (*Cx3cr1<sup>EGFP/+</sup>*). The vasculature was labeled with an antibody against platelet endothelial cell  
402 adhesion molecule (PECAM). To start, we focused our analyses in the frontal cortex.  
403 Juxtavascular microglia were defined as microglia with at least 30% of their soma perimeter in  
404 contact with blood vessels and soma centers that were within 10µm of the vessel, which we  
405 confirmed with orthogonal views and 3D surface rendering (Fig 1. A-F; See also Movies 1 and  
406 2). Juxtavascular microglia were further distinguished from perivascular macrophages by their  
407 morphology with processes emanating from their soma and higher levels of EGFP. Using these  
408 criteria, we found a higher percent of the total microglial population were juxtavascular at P1-P5  
409 (Fig. 1G) in the frontal cortex, which was independent of sex (data not shown). The percent  
410 association dropped to below 20% by P14 and was maintained at later ages. We confirmed that  
411 this developmental regulation of juxtavascular microglia was independent of changes in  
412 vasculature density over development. While the total vascular content of the cortex increases  
413 as the brain grows, the density of the blood vessels within a given field of view is unchanged

414 across development (Fig. 1H). Consistent with the results in mouse, the ventricular and  
415 subventricular zones of the prenatal human brain at the level of the frontal cortex also showed a  
416 high percent of juxtavascular microglia. This association in the developing human brain peaked  
417 at 18-24 gestational weeks (GW) where 38% of total microglia were juxtavascular (Fig. 1I-J)—a  
418 percentage similar to what we identified in early postnatal mice. Together, these data  
419 demonstrate that a large percentage of the total microglia are juxtavascular in the early  
420 postnatal mouse and prenatal human brain.

421  
422 **Juxtavascular microglia are largely associated with capillaries in the early postnatal**  
423 **cortex**

424 While previous work has described similar high association of microglia with the  
425 vasculature in the embryonic/prenatal brain, these studies did not use markers to distinguish  
426 microglia from perivascular macrophages (Fantin et al. 2010; Monier et al. 2007; Checchin et al.  
427 2006). Therefore, we next sought to confirm that vascular-associated EGFP-positive cells were,  
428 indeed, microglia versus perivascular macrophages and determine which types of vessels were  
429 being contacted by microglia. We found that the juxtavascular EGFP+ cells that we initially  
430 identified as microglia based on their larger numbers of processes and higher levels of EGFP  
431 (Fig. 1; Fig. 2A-B filled arrowheads) were also positive for the microglia-specific marker P2RY12  
432 (Fig. 2A, filled arrowhead) and negative for the perivascular macrophage-specific marker LYVE1  
433 (Fig. 2B, unfilled arrowheads) (Butovsky et al. 2014; Zeisel et al. 2015). Using anti-P2RY12 to  
434 label microglia in wild-type mice or EGFP in *Cx3cr1*<sup>EGFP/+</sup> mice, which are heterozygote for  
435 CX3CR1, we obtained similar percentages of juxtavascular microglial and vascular density (Fig.  
436 2C-D), confirming results were independent of the microglial labeling technique. We also found  
437 that these juxtavascular microglia were associated largely along unsegmented vessels, rather  
438 than branch points, across postnatal development (Fig. 2E). We next assessed what types of  
439 vessels were contacted by juxtavascular microglia, using a combination of parameters.



440 Capillaries are  $\leq 8$   $\mu\text{m}$  in diameter and are smooth muscle actin (SMA)-negative and Platelet  
441 Derived Growth Factor Receptor  $\beta$  (PDGFR $\beta$ )-positive (Grant et al. 2019; Mastorakos and  
442 MCGavern 2019). Arterioles are  $> 8$   $\mu\text{m}$  in diameter and are SMA-positive and a subset of pre-  
443 capillary arterioles are also PDGFR $\beta$ -positive (Grant et al. 2019). Using these markers, we  
444 identified that juxtavascular microglia were largely contacting capillaries ( $\leq 8$   $\mu\text{m}$ , SMA-negative,  
445 PDGFR $\beta$ -positive; Fig. 2F-H). These experiments establish that a large percentage of bona fide  
446 microglia are associated with unsegmented capillaries in the postnatal cerebral cortex and these  
447 percentages are similar in wild type and *Cx3cr1*<sup>EGFP/+</sup> mice.

448  
449 **High percentages of juxtavascular microglia occur when microglia are actively colonizing**  
450 **the cortex in a CX3CR1-dependent manner**

451 Over development, microglia undergo a dynamic process of colonization and expansion  
452 in a rostral-to-caudal gradient (Ashwell 1991; Perry, Hume, and Gordon 1985). Similar to  
453 previously published work (Nikodemova et al. 2015), we identified a large expansion in cortical  
454 microglia between P1 and P14, with microglia colonizing the more rostral frontal cortex region  
455 prior to the more caudal somatosensory cortex (Fig. 3A-C, bar graphs in B-C). Microglia-  
456 vascular association mirrored this rostral-to-caudal gradient by which microglia colonize the  
457 brain with a higher percentage of juxtavascular microglia at P1-P5 (46.3% at P1 and 44.4% at  
458 P5) in the frontal cortex and at P5-P7 (39.1% at P5 and 34.2% at P7) in the more caudal  
459 somatosensory cortex (Fig. 3B-C, line graphs). Moreover, during times of active microglial  
460 colonization in both postnatal cortical regions (P1-P5 in the frontal cortex and P1-P7 in the  
461 somatosensory cortex), significantly more microglial primary processes were aligned parallel  
462 with vessels compared to older ages (Fig. 3D-G). This parallel juxtavascular microglial  
463 orientation along vessels is consistent with a migratory orientation.

464 To further investigate microglia-vascular interactions in the context of colonization of the  
465 postnatal cortex, we assessed a somatosensory sub-region where the pattern of microglial



466 colonization has been well described—the barrel cortex. Layer IV of the barrel cortex contains  
467 thalamocortical synapses, which form a highly precise synaptic map of the vibrissae (whiskers)  
468 on the snout. These layer IV thalamocortical synapses form discrete barrel structures  
469 corresponding to each whisker, which are separated by septa where thalamocortical synapses  
470 are largely absent (Fig. 4A) (Woolsey and Van der Loos 1970; Welker and Woolsey 1974).  
471 Previous work has shown that microglia first localize to the septa and then colonize these  
472 thalamocortical synapse-dense barrel centers between P6 and P7 and this process is delayed  
473 to P8-P9 day in CX3CR1-deficient (*Cx3cr1*<sup>-/-</sup>) mice (Hoshiko et al. 2012). This delay in  
474 recruitment in *Cx3cr1*<sup>-/-</sup> mice is concomitant with a delay in synapse maturation. However, it was  
475 unclear how CX3CR1 was regulating the timing of microglial recruitment to synapses in the  
476 barrel cortex. To identify barrels, we labeled thalamocortical presynaptic terminals with an  
477 antibody against vesicular glutamate transporter 2 (VGLUT2). Microglia were labeled with  
478 transgenic expression of EGFP in either *Cx3cr1*<sup>+/-</sup> (*Cx3cr1*<sup>EGFP/+</sup>) or *Cx3cr1*<sup>-/-</sup> (*Cx3cr1*<sup>EGFP/EGFP</sup>)  
479 mice. The vasculature was labeled with anti-PECAM. Similar to previous work (Hoshiko et al.  
480 2012), microglia infiltrated thalamocortical synapse-dense barrel centers (outlined with a yellow  
481 dotted line in Fig. 4C-F) from the septa by P6-P7 in *Cx3cr1*<sup>+/-</sup> mice and this process was  
482 delayed by one day in *Cx3cr1*<sup>-/-</sup> mice (Fig. 4B-D). Strikingly, just prior to entering barrel centers  
483 at P5-P6 in *Cx3cr1*<sup>+/-</sup> mice, a higher percentage of microglia were juxtavascular (Fig. 4E, G,  
484 arrowheads). Further, this microglia-vascular association was delayed by one day in *Cx3cr1*<sup>-/-</sup>  
485 mice (Fig. 4F-G), which is consistent with the delay in microglial migration into barrel centers in  
486 these mice (Fig. 4B). In both genotypes, the percentage of juxtavascular microglia decreased  
487 once the microglia began to colonize the thalamocortical synapse-dense barrel centers, P7 in  
488 *Cx3cr1*<sup>+/-</sup> mice and P8 in *Cx3cr1*<sup>-/-</sup> mice (Fig. 4F-G). These changes in microglia-vascular  
489 interactions were independent of any changes in total microglial or vascular density in layer IV  
490 (Fig. 4H-I), but rather specific to microglial distribution between the septa and barrels. These  
491 data are consistent with a model by which microglia use the vasculature to colonize synapse-

492 dense cortical regions at the appropriate developmental timing. They further suggest that  
493 CX3CR1 signaling modulates the timing of microglial-vascular interactions and, subsequently,  
494 colonization to synapse-dense regions of the barrel cortex.

495

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

498 With data demonstrating that high percentages of microglia are juxtavascular when they  
499 are actively colonizing the brain with processes aligned parallel to the vessel, we next  
500 performed live imaging to assess migration. As the early postnatal cortex is challenging to  
501 image *in vivo*, we performed our initial analyses in acute cortical slices. Acute slices of  
502 somatosensory cortex were prepared from early postnatal (P7) and adult (P $\geq$ 120) *Cx3cr1*<sup>EGFP/+</sup>  
503 mice, which were given a retro-orbital injection of Texas Red labeled dextran to label blood  
504 vessels prior to slice preparation. We then imaged microglia every 5 minutes over 6 hours at  
505 both ages (Fig. 5A). Live imaging at P7 revealed significant juxtavascular microglial soma  
506 movement along blood vessels in the somatosensory cortex compared to vascular-  
507 unassociated microglia at P7 (Fig. 5B, D, see also Movies 3-5). Specifically, 28.6% of  
508 juxtavascular microglia somas moved at a rate of 3-5 $\mu$ m/hour and another 26.1% moved at a  
509 rate of 5-7.5 $\mu$ m/hour (Fig. 5D). In comparison, only 9.3% and 6.8% of vascular-unassociated  
510 microglia at the same age moved at 3-5 $\mu$ m/hour and 5-7.5 $\mu$ m/hour, respectively. We further  
511 found that when we assessed just the motile soma at P7, significantly more juxtavascular  
512 microglia somas travelled >20 $\mu$ m (30.9% traveled 20-30 $\mu$ m and 23.6% traveled 30-45 $\mu$ m) over  
513 6 hours compared to vascular-unassociated microglia (7.5% and 6.8% traveled 20-30 $\mu$ m and  
514 30-45 $\mu$ m, respectively) (Fig. 5E). Importantly, the juxtavascular microglia soma velocities and  
515 distances traveled are consistent with the rate and distances at which microglia migrate to barrel  
516 centers within the somatosensory cortex *in vivo* where the distance between the septa and  
517 barrel center is ~80 $\mu$ m and it takes ~24 hours for microglia to reach the barrel center from the

518 septa. Demonstrating directional motility and suggesting migration along the vessel, 84.1% of  
519 these postnatal juxtavascular microglia had a motility trajectory of  $\leq 15^\circ$  along the blood vessel  
520 (Fig. 5F). Together, these data demonstrate directional migration of juxtavascular microglia at  
521 distances and speeds consistent with colonization of the cortex (P7).

522 Interestingly, this migratory behavior along the vasculature was developmentally  
523 regulated and juxtavascular microglia in adult slices were largely stationary (Fig. 5C-D; see also  
524 Movie 6). We further confirmed the stationary phenotype of juxtavascular microglia in the adult  
525 cortex by *in-vivo* 2-photon live imaging in *Cx3cr1<sup>EGFP/+</sup>* mice. Windows were placed over the  
526 visual cortex, which was most conducive to our head posts necessary for stabilizing the head in  
527 awake, behaving mice during imaging. We have found similar microglia-vascular interactions by  
528 static confocal imaging in the visual cortex (data not shown). Mice were given a retro-orbital  
529 injection of Texas Red labeled dextran to label blood vessels prior to imaging and juxtavascular  
530 microglia were imaged every 5 min over the course of 2 hours (Fig. 5G). As observed in acute  
531 cortical slices, 100% of juxtavascular and vascular-unassociated microglia were stationary (Fig.  
532 5H; see also Movie 7). To further understand long-term dynamics, we imaged juxtavascular  
533 microglia *in vivo* over the course of 6 weeks (Fig. 5I). We identified that 82.9% of juxtavascular  
534 microglia present on day 0 of imaging remained on the vasculature 6 weeks later (Fig. 5J-K).  
535 Together, these data demonstrate that juxtavascular microglia in the postnatal cortex are highly  
536 migratory compared to non-vascular associated microglia. In contrast, juxtavascular microglia in  
537 adulthood are largely stationary, which suggests the establishment of a niche for juxtavascular  
538 microglia in the adult brain.

539

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

541 Our data demonstrate a strong microglial association and migration along the developing  
542 postnatal cortical vasculature. One possible mechanism regulating these developmental  
543 changes in juxtavascular microglia is the changing cellular composition of the NVU over

544 development. The neurovascular unit (NVU) begins to form during embryonic development,  
545 when pericytes associate with endothelial cells. Later in postnatal development, astrocytes are  
546 born and begin wrapping their endfeet around vessels until the vast majority of the vasculature  
547 is ensheathed by astrocyte endfeet by adulthood (Daneman et al. 2010; Schiweck, Eickholt, and  
548 Murk 2018; Bayraktar et al. 2015). As previously described (Daneman et al. 2010), the territory  
549 of Aquaporin 4 (AQP4)-positive astrocyte endfeet on PDGFR $\beta$ + capillaries was low in the early  
550 postnatal cortex and then expanded over the first postnatal week (Fig. 6 A-D, bar graph in D). In  
551 more mature animals ( $\geq$ P21), astrocytic endfeet covered  $\sim$ 85% of vessels in the frontal cortex.  
552 Intriguingly, this developmental timing of astrocyte endfoot coverage mirrored the  
553 developmental shift in the percentage of juxtavascular microglia in the cortex (Fig. 6D, line  
554 graph). That is, as the percentage of juxtavascular microglia decreased, astrocyte endfoot  
555 coverage increased. This astrocyte coverage also correlated with the timing of decreased  
556 microglial motility along the vessels (Fig. 5). We further assessed microglia-astrocyte endfoot  
557 interactions by confocal microscopy and 3D surface rendering. At all ages, microglia only  
558 contacted the vasculature in areas either completely void of astrocyte endfeet or in areas where  
559 vessels were not fully covered by the endfeet (Fig. 6 A-C, white arrow heads; Fig. 6E, see also  
560 Movies 8-10). Juxtavascular microglia were never in direct contact with solely astrocyte endfeet  
561 and no vessel at any age assessed (Fig. 6E). Given that cells of the NVU are nanometers apart  
562 from each other, we confirmed these results with expansion microscopy (ExM; Fig 6F-G),  
563 structured illumination microscopy (SIM; Fig. 6H-I) and electron microscopy (EM; Fig 7). By EM,  
564 microglia were identified based on characteristic microglial morphologies. Microglia nuclei tend  
565 to be half-mooned shape or long and thin with electron dense heterochromatin around the edge  
566 of the nucleus. Microglia were further distinguished by EM from perivascular macrophages by  
567 having processes emanating from the soma. Serial sectioning and 3D reconstruction of a  
568 representative cell captured by EM from each age confirmed that juxtavascular microglia  
569 contacted the basal lamina in vascular areas without full astrocyte endfoot coverage at all ages

570 (Fig. 7C, see also Movies 11 and 12). Together, these data demonstrate that juxtavascular  
571 microglia associate with the vascular basal lamina and associate with the vasculature in areas  
572 lacking full coverage by astrocyte endfeet. The data raise the intriguing possibility that lack of  
573 astrocyte endfeet in early postnatal development provides a permissive environment for  
574 juxtavascular microglial association with and migration along the vasculature as they colonize  
575 the brain.

576

## 577 **DISCUSSION**

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

593

## 594 **A possible role for the vasculature in regulating microglial colonization**

595 Microglia are born as primitive macrophages in the embryonic yolk sac and enter the  
596 neuroepithelium at embryonic day E9.5 by crossing the pial surface and lateral ventricles  
597 (Navascués et al. 2000; Swinnen et al. 2013; Ginhoux et al. 2010). Microglia then migrate and  
598 proliferate through the brain parenchyma in a rostral-to-caudal gradient to colonize the  
599 embryonic brain (Sorokin et al. 1992; Navascués et al. 2000; Swinnen et al. 2013; Alliot, Godin,  
600 and Pessac 1999; Perry, Hume, and Gordon 1985; Ashwell 1991). Signaling mechanisms have  
601 been identified to regulate initial microglial infiltration into the brain parenchyma, such as matrix  
602 metalloproteinases (MMPs), stromal cell derived factor 1 (SDF-1), and Cxcl12/Cxcr4 signaling  
603 (Ginhoux et al. 2010; Arno et al. 2014; Ueno and Yamashita 2014). However, far less is known  
604 about the mechanisms regulating microglial localization to the appropriate brain regions once  
605 they reach the parenchyma, particularly during postnatal development. Previous work has  
606 shown microglia can migrate along the vasculature in acute embryonic brain slices and brain  
607 slices prepared from postnatal mice in an injury context (Smolders et al. 2017; Grossmann et al.  
608 2002). In addition, other work has shown that oligodendrocyte precursor cells (OPCs) require  
609 the vasculature as a physical substrate for migration (Tsai et al. 2016). Similar findings have  
610 been identified for neural stem cells where the timing of astrocyte endfeet to the vessels has  
611 also been implicated (Bovetti et al. 2007; Fujioka, Kaneko, and Sawamoto 2019; Whitman et al.  
612 2009). We have identified that microglia are highly associated with vasculature during the peak  
613 of microglial colonization and recruitment to synapses. Furthermore, these vascular-associated  
614 microglia are migratory along blood vessels during early postnatal development and later  
615 become stationary once microglial colonization is complete. We also show in CX3CR1-deficient  
616 mice with known delays in microglial colonization of synapse-dense cortical regions that there  
617 are concomitant delays in microglial association with the vasculature. As we have observed no  
618 significant expression of *Cx3c1* (the CX3CR1 ligand) by vascular cells (Gunner et al. 2019) and  
619 a subset of microglia still associate with the vasculature in *Cx3cr1*<sup>-/-</sup> mice, this delay in microglial  
620 vascular association in *Cx3cr1*<sup>-/-</sup> mice is most likely due to disruptions in chemokine gradient

621 signaling from neuronal sources of CX3CL1 versus a direct effect of vascular adhesion. This  
622 would suggest that microglia receive directional cues from surrounding cells, use the  
623 vasculature as a physical substrate to migrate towards those cues, and the timing of this  
624 migration along the vasculature is regulated by CX3CR1. As *Cx3cr1*<sup>-/-</sup> mice have delays in  
625 synapse maturation and pruning and, long-term, have behavioral deficits consistent with an  
626 autism-like phenotype, it suggests that these microglia-vascular associations in development  
627 have long-term consequences (Paolicelli et al. 2011; Zhan et al. 2014; Hoshiko et al. 2012). The  
628 vascular cues regulating microglial adhesion and migration in the healthy CNS are yet to be  
629 identified, which will be key to determine the relative importance of microglia-vascular  
630 interactions for microglial colonization, brain development, and long-term CNS function.

631

### 632 **Microglia-astrocyte interactions at the NVU interface**

633 Another interesting direction is to determine the role of astrocyte endfeet in regulating  
634 microglia-vascular interactions. Astrocytes are born and begin wrapping their processes to form  
635 endfeet along blood vessels during the first postnatal week (Daneman et al. 2010). By  
636 adulthood, astrocytes endfeet ensheath 60-95% of the vasculature (Mathiisen et al. 2010;  
637 Korogod, Petersen, and Knott 2015). Here, we demonstrate that juxtavascular microglia in the  
638 postnatal cortex represent a large percentage of total microglia and are migratory along the  
639 vasculature. Juxtavascular microglia migration decreases as astrocyte endfeet develop and  
640 ensheath the vasculature. In addition, we showed that microglia contact vessels at all ages in  
641 areas lacking full astrocytic endfoot coverage and EM revealed high association between  
642 juxtavascular microglia and the vascular basal lamina. These data raise the intriguing possibility  
643 that the basal lamina provides an adhesive substrate for microglial association and migration,  
644 which becomes restricted upon astrocyte endfoot arrival. Astrocyte endfeet may, therefore,  
645 physically exclude microglia from contacting the basal lamina and associating with the  
646 vasculature. Another possibility is that microglia in the postnatal brain repel astrocyte endfeet,



647 but this repellent signal later decreases as the animal matures so that astrocyte endfeet can  
648 wrap the vessels. Analysis of astrocyte endfoot-juxtavascular microglia interactions along blood  
649 vessels will be important going forward.

650

### 651 **Possible functions for juxtavascular microglia in the healthy CNS**

652 Are juxtavascular microglia a unique subpopulation of microglial cells that perform  
653 distinct functions at the NVU? Evidence in the literature suggests microglia play important roles  
654 in regulating the vasculature, but it is unclear if these functions are specific to juxtavascular  
655 microglia. For example, in the embryonic brain, microglia are often localized to vascular junction  
656 points and depletion of all microglia is associated with a decrease in vascular complexity (Fantin  
657 et al. 2010). Similar findings have been identified in the developing retina (Rymo et al. 2011;  
658 Checchin et al. 2006; Dudiki et al. 2020). Our data demonstrating that microglia are localized to  
659 the vasculature prior to the arrival of the astrocyte endfeet could place microglia in a position to  
660 regulate fine-scale remodeling of the vasculature throughout the brain and/or help to maintain  
661 the BBB prior to astrocyte endfoot arrival. Arguing against the latter, microglia depletion during  
662 development does not appear to induce changes in BBB integrity in the postnatal brain  
663 (Parkhurst et al. 2013; Elmore et al. 2014). These data are in contrast to the inflamed adult  
664 CNS, where microglia regulate BBB integrity (Zhao et al. 2018; Stankovic, Teodorczyk, and  
665 Ploen 2016). One of the most recent studies shows that during systemic inflammation,  
666 parenchymal microglia migrate to the vasculature and help to maintain the BBB at acute stages  
667 (Haruwaka et al. 2019). However, with sustained inflammation, microglia phagocytose astrocyte  
668 endfeet and facilitate BBB breakdown. In the absence of inflammation, it remains unknown what  
669 functions juxtavascular microglia may perform. However, our *in vivo* live imaging data  
670 demonstrating microglia in the adult brain are stationary for nearly 2 months opens up the  
671 possibility that these cells reside in a vascular niche to perform specialized functions. One  
672 possible role could be to serve as immune surveillant “first responders”. We report that microglia



673 are in direct contact with the basal lamina at all ages in areas lacking astrocyte endfeet, which  
674 typically work to maintain the BBB (Abbott, Rönnbäck, and Hansson 2006; Kimelberg and  
675 Nedergaard 2010; Macvicar and Newman 2015). As a result, juxtavascular microglia in the  
676 healthy brain are in an ideal location to serve as conduits to relay changes in the peripheral  
677 environment (changes in the microbiome, infection, etc.) to the CNS. Indeed, many of these  
678 changes in peripheral immunity are known to directly impact neural circuit function and behavior  
679 in ways we do not yet fully understand (Hanamsagar and Bilbo 2017; Lebovitz et al. 2018;  
680 Abdel-haq et al. 2018) and recent work in the inflamed brain has demonstrated microglia can  
681 serve as a conduit by which the microbiome affects neuroinflammation (Rothhammer et al.  
682 2018).

683

#### 684 **Microglia-vascular interactions: Implications for CNS disease**

685 Our findings have important implications for neurological diseases associated with the  
686 injured or aged CNS where there is enhanced microglial association with the vasculature, such  
687 as in stroke, brain tumors, multiple sclerosis (MS), and Alzheimer's disease (Stankovic,  
688 Teodorczyk, and Ploen 2016; Zhao et al. 2018). This enhanced association can lead to further  
689 breakdown of the BBB and infiltration of peripheral immune cells into the CNS and possibly  
690 angiogenesis in the case of brain tumors (Stankovic, Teodorczyk, and Ploen 2016; Zhao et al.  
691 2018; Haruwaka et al. 2019). Therefore, understanding precisely when and where microglia  
692 interact with the vasculature in the healthy brain may lead to therapeutic strategies to reduce  
693 vascular pathology and facilitate recovery. One intriguing possibility is that these sites of  
694 microglial contact, which lack astrocyte endfeet, are more vulnerable to BBB breakdown and  
695 infiltration of peripheral immune cells and factors. In addition to neurodegenerative disorders,  
696 our findings may also have important implications for neurodevelopmental disorders such as  
697 autism spectrum disorders (ASDs). For example, microglia-vascular interactions may be  
698 important for the timing of microglial colonization to synapse-dense brain regions where they

699 regulate synapse maturation and pruning during critical windows in development (Paolicelli et al.  
700 2011; Hoshiko et al. 2012; Tremblay, Lowery, and Majewska 2010; Schafer et al. 2012; Gunner  
701 et al. 2019). If these interactions are disrupted, the timing of synapse development and,  
702 ultimately, neural circuit function may be altered. This is supported by our data from *Cx3cr1*<sup>-/-</sup>  
703 mice showing delays in microglial association with the vessels, which is concomitant with known  
704 delays in microglial recruitment to developing synapses and delays in synapse maturation in  
705 these mice (Paolicelli et al. 2011; Zhan et al. 2014; Hoshiko et al. 2012). Long term, *Cx3cr1*<sup>-/-</sup>  
706 mice have phenotypes associated with ASD, including decreased functional brain connectivity,  
707 deficits in social interactions, and increased repetitive behaviors (Zhan et al. 2014). However, a  
708 better understanding of how vascular interactions affect microglial colonization and extending  
709 these analyses of microglia-vascular interactions into the ASD human brain will be necessary.

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

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959 **FIGURE LEGENDS**

960 **Figure 1: A high percentage of microglia are juxtavascular during early postnatal**  
961 **development. A-B.** Representative low magnification tiled images of microglia (green, EGFP)  
962 associated with vasculature (magenta, anti-PECAM) in the P5 (**A**) and P28 (**B**) frontal cortex.  
963 Filled arrowheads denote juxtavascular microglia. Scale bars= 100  $\mu\text{m}$  (**A**) and 50  $\mu\text{m}$  (**B**). **C-D.**  
964 High magnification, orthogonal view (**C**) and 3D reconstruction and surface rendering (**D**) of  
965 juxtavascular microglia in the P5 frontal cortex (see also Movie 1). Scale bars= 10  $\mu\text{m}$ . **E-F.**  
966 Orthogonal (**E**) and 3D reconstruction and surface rendering (**F**) of a juxtavascular microglia in  
967 the P28 frontal cortex (see also Movie 2). Scale bars= 10  $\mu\text{m}$ . **G.** The percent of the total  
968 microglia population associated with vasculature over development in the frontal cortex. One-  
969 way ANOVA with Dunnett's post hoc; comparison to P $\geq$ 21, n=4 littermates per developmental  
970 time point, \*\*\*\*p<.0001. **H.** Vascular density over development in the frontal cortex. One-way  
971 ANOVA with Dunnett's post hoc; comparison to P $\geq$ 21, n=4 littermates per developmental time  
972 point. **I.** Representative image of microglia (green, anti-IBA1) associated with vasculature  
973 (magenta, anti-CD31) in gestational week (GW) 24 in the ventricular zone (VZ) and  
974 subventricular zone (SVZ) at the level of the human frontal cortex. Filled arrowheads denote  
975 juxtavascular microglia. Scale bar=20  $\mu\text{m}$ . **J.** Quantification of the percentage of total microglia  
976 associated with vasculature in the human brain. One-way ANOVA across all ages, p=0.0544,  
977 n=1 specimen per gestational age. All error bars represent  $\pm$  SEM.

978  
979 **Figure 2: Juxtavascular microglia predominantly contact capillaries in the postnatal**  
980 **cortex. A.** A representative image of a juxtavascular microglia (filled arrowhead) in the P5  
981 frontal cortex. Microglia are labeled using the *Cx3cr1*<sup>EGFP/+</sup> reporter mouse (green; **Ai**) and  
982 immunolabeling for a microglia-specific marker anti-P2RY12 (red; **Aii**). The vasculature is  
983 labeled with anti-PECAM (magenta) in the merged image (**Aiii**). Scale bar= 10 $\mu\text{m}$ . **B.** A  
984 representative image of LYVE1-negative microglia (green, EGFP, filled arrowheads) and

985 LYVE1-positive perivascular macrophages (gray, anti-LYVE1, unfilled arrowheads) associated  
986 with vasculature (magenta, anti-PECAM) in the P5 frontal cortex. Scale bar= 10 $\mu$ m. **C.**  
987 Quantification of juxtavascular microglia across development labeled either with EGFP in  
988 *Cx3cr1*<sup>EGFP/+</sup> mice (black bars) or anti-P2RY12 in wild type mice (WT, white bars) frontal  
989 cortices. Two-way ANOVA with a Sidak's post hoc; n=3-4 littermates per genotype per  
990 developmental time point. **D.** Quantification of vascular density in *Cx3cr1*<sup>EGFP/+</sup> (black bars) and  
991 WT (white bars) frontal cortices over development. Two-way ANOVA with a Sidak's post hoc;  
992 n=3-4 littermates per genotype per developmental time point. **E.** Quantification of the percent of  
993 juxtavascular microglia contacting branched (black bars) or unsegmented (gray bars) vessels.  
994 Two-way ANOVA with a Sidak's post hoc; n=3-4 littermates per developmental time point,  
995 \*p<.05, \*\*\*p<.001, \*\*\*\*p<.0001. **F.** A representative image of a juxtavascular microglia (green,  
996 EGFP, filled arrowhead) contacting smooth muscle cell actin (gray, SMA)-negative capillaries  
997 (magenta; PDGFR $\beta$ ) in the P5 frontal cortex. Scale bar= 10 $\mu$ m **G.** Quantification of the percent  
998 of juxtavascular microglia contacting SMA-positive or -negative vessels at P5 and P $\geq$ 21 in the  
999 frontal cortex. Two-way ANOVA with a Sidak's post hoc; n=3 littermates per genotype per  
1000 developmental time point, \*\*\*\*p<.0001. **H.** Quantification of the percent of juxtavascular  
1001 microglia contacting vessels  $\leq$ 8 $\mu$ m and  $>$ 8 $\mu$ m at P5 and P $\geq$ 21 in the frontal cortex. Two-way  
1002 ANOVA with a Sidak's post hoc; n=4 littermates per genotype per developmental time point,  
1003 \*\*\*\*p<.0001. All error bars represent  $\pm$  SEM.

1004  
1005 **Figure 3: Microglia associate and align with vasculature as they colonize the cortex in a**  
1006 **rostral-to-caudal gradient. A.** Tiled sagittal sections of a P1 (**Ai**), P7 (**Aii**), and P14 (**Aiii**)  
1007 *Cx3cr1*<sup>EGFP/+</sup> brain. The dotted yellow and red lines outline the frontal and somatosensory cortex,  
1008 respectively. Scale bars= 400 $\mu$ m. **B-C.** Left Y axis and gray bars: quantification of microglial  
1009 density over development in the frontal cortex (**B**) and somatosensory cortex (**C**). One-way  
1010 ANOVA with Dunnett's post hoc; comparison to P $\geq$ 21, n=4 littermates per developmental time

1011 point, \* $p < .05$ , \*\* $p < .01$ , \*\*\*\* $p < .0001$ . Right Y axis and black line graphs: the percent of the total  
1012 microglia population associated with vasculature over development in the frontal cortex (**B**) and  
1013 somatosensory cortex (**C**). Note, data corresponding to the percent of juxtavascular microglia in  
1014 the frontal cortex (line graph in C) are the same as presented in Fig. 1G. One-way ANOVA with  
1015 Dunnett's post hoc; comparison to  $P \geq 21$ ,  $n=4$  littermates per developmental time point,  
1016 ++++ $p < .0001$ . **D-E**. Representative images of juxtavascular microglia (EGFP, green in **Di** and  
1017 **Ei**; black in **Dii** and **Eii**) primary processes aligned parallel (**D**) with vessels (magenta, anti-  
1018 PECAM) in the P5 frontal cortex, which were largely not aligned at P28 (**E**). Filled arrowheads  
1019 denote processes aligned parallel to the vessel and unfilled arrowheads denote those microglial  
1020 processes that are not aligned with the vessel. The dotted magenta line in **Dii** and **Eii** outline the  
1021 vessel in **Di** and **Ei**. Scale bars= 10 $\mu$ m. **F-G**. Quantification of the percent of juxtavascular  
1022 primary processes that are aligned parallel with vessels in the frontal (**F**) and somatosensory  
1023 (**G**) cortices over development. One-way ANOVA with Dunnett's post hoc; comparison to  $P \geq 21$ ,  
1024  $n=3-4$  littermates per developmental time point, \*\*\*  $p < .001$ , \*\*\*\* $p < .0001$ . All error bars represent  
1025  $\pm$  SEM.

1026  
1027 **Figure 4: A high percentage of microglia associate with vasculature as they are recruited**  
1028 **to synapses in the cortex in a CX3CR1-dependent manner. Ai-Aii.** Layer IV of the barrel  
1029 cortex contains thalamocortical synapses, which form a highly precise synaptic map of the  
1030 vibrissae (whiskers) on the snout. **Aiii.** A low magnification representative image of a tangential  
1031 section through layer IV of the barrel cortex shows layer IV thalamocortical presynaptic  
1032 terminals (red, anti-VGluT2), form discrete barrel structures corresponding to each whisker,  
1033 which are separated by septa where thalamocortical terminals are largely absent. Microglia are  
1034 labeled by EGFP (green) and the vasculature is labeled with anti-PECAM (gray). White box  
1035 denotes a single barrel. Scale bar= 100 $\mu$ m. **B.** Quantification of the number of microglia per  
1036  $\text{mm}^2$  within the barrel centers in developing *Cx3cr1*<sup>+/-</sup> (black bars) and *Cx3cr1*<sup>-/-</sup> (gray bars)

1037 mice. Two-way ANOVA with a Sidak's post hoc; n=4 littermates per genotype per  
1038 developmental time point; \*\* p<.01, \*\*\*p<.001. **C-D.** Representative images of quantification in  
1039 B. Images are zoomed in to show single barrels within tangential sections of layer IV of the  
1040 barrel cortex (denoted by white box in Aiii) where microglia (green) are recruited to barrel  
1041 centers in *Cx3cr1*<sup>+/-</sup> by P7 (**C**) and in *Cx3cr1*<sup>-/-</sup> by P8 (**D**). Asterisks denote microglia located  
1042 within barrel centers. The dotted yellow lines denote the perimeters of the VGluT2-positive  
1043 thalamocortical inputs (red), which define the barrels vs. the septa. Scale bars= 30µm. **E-F.** The  
1044 same representative fields of view in C-D but lacking the anti-VGluT2 channel and, instead,  
1045 including the channel with anti-PECAM immunostaining (magenta) to label the vessels.  
1046 Microglia are still labeled with EGFP (green). Dotted yellow lines still denote the perimeters of  
1047 the VGluT2-positive barrels (red in C-D). Juxtavascular microglia in *Cx3cr1*<sup>+/-</sup> and *Cx3cr1*<sup>-/-</sup> mice  
1048 are denoted by filled arrowheads. Scale bar= 30µm. **G.** Quantification of the percent of microglia  
1049 associated with the vasculature in *Cx3cr1*<sup>+/-</sup> (black lines) and *Cx3cr1*<sup>-/-</sup> (gray lines) animals over  
1050 development in layer IV of the barrel cortex demonstrates a peak of vascular association in  
1051 *Cx3cr1*<sup>+/-</sup> mice at P5-P6, which is delayed to P7-P8 in *Cx3cr1*<sup>-/-</sup> coincident with delayed  
1052 microglial recruitment to barrel centers. Two-way ANOVA with a Tukey's post hoc; n=4-5  
1053 littermates per genotype per developmental time point; \*p<.05, \*\*\*p<.001, compared to P9  
1054 *Cx3cr1*<sup>+/-</sup>. **H-I.** Quantification of microglial (**H**) and vascular (**I**) density in *Cx3cr1*<sup>+/-</sup> (black bars)  
1055 and *Cx3cr1*<sup>-/-</sup> (gray bars) animals over development in layer IV of the barrel cortex. Two-way  
1056 ANOVA with a Sidak's post hoc; n=4 littermates per genotype per developmental time point. All  
1057 error bars represent ± SEM.

1058  
1059 **Figure 5: Juxtavascular microglia migrate along blood vessels as they colonize the**  
1060 **developing brain and are largely stationary in adulthood. A.** A schematic of the live imaging  
1061 experiment. *Cx3cr1*<sup>EGFP/+</sup> mice received a retro-orbital injection of Texas red labeled dextran to  
1062 label the vasculature 10 minutes prior to euthanasia. Coronal somatosensory cortices were cut



1063 and imaged every 5 minutes over 6 hours immediately following slice preparation. **B-C.**  
1064 Representative fluorescent images from a 6-hour live imaging session from a P7 (**B**) and P $\geq$ 120  
1065 (**C**) slice. Filled arrowheads indicate microglial soma position at t=0. Unfilled arrowheads  
1066 indicate the location of the same microglial soma at 0hr (**Bi, Ci**), 1hrs (**Bii, Cii**), 2hrs (**Biii, Ciii**),  
1067 3hrs (**Biv, Civ**), 4hrs (**Bv, Cv**), 5hrs (**Bvi, Cvi**), and 6hrs (**Bvii, Cvii**). See also Movies 3-6.  
1068 Scale bars= 30 $\mu$ m. **D.** Quantification of juxtavascular (black bars) and vascular-unassociated  
1069 (gray bars) microglia soma motility speed/velocity. Two-way ANOVA with a Sidak's post hoc;  
1070 n=4 mice per time point; \*\*p<.01, \*\*\*p<.001. **E.** Quantification of the distance traveled of  
1071 juxtavascular (black bars) and vascular-unassociated (gray bars) microglia somas in the P7  
1072 somatosensory cortex. Two-way ANOVA with a Sidak's post hoc; n=4 mice; \*\*p<.01, \*\*\*p<.001,  
1073 \*\*\*\*p<.0001. **F.** Quantification of migratory juxtavascular microglia trajectory angles in the P7  
1074 somatosensory cortex. Unpaired student's t-test; n=4 mice per time point; \*\*\*\*p<.0001. **G.** A  
1075 schematic of short-term 2-photon live imaging experiment in adult cortex. *Cx3cr1*<sup>EGFP/+</sup> mice  
1076 received a retro-orbital injection of Texas Red-labeled dextran to visualize the vasculature 10  
1077 min prior to each imaging session. EGFP+ juxtavascular microglia were then imaged every 5  
1078 minutes for 2 hours. See also Movie 7. **H.** Quantification of the percent of juxtavascular (black  
1079 bars) and vascular-unassociated (gray bars) microglia that remain stationary for 2 hours.  
1080 Unpaired student's t-test; n=3 mice per developmental time point. **I.** A schematic of the long-  
1081 term 2-photon live imaging experiment in adult visual cortex. *Cx3cr1*<sup>EGFP/+</sup> mice received a retro-  
1082 orbital injection of Texas Red-labeled dextran to visualize the vasculature 10 min prior to each  
1083 imaging session. EGFP+ juxtavascular microglia were then imaged for 6 weeks. **J.**  
1084 Quantification of the percent of juxtavascular microglia on vessels on day 0 that remain on  
1085 vessels through six weeks of imaging. Data are representative of n=3 mice. **K.** Representative  
1086 fluorescent images acquired during a 6-week live imaging session from a single mouse. Filled  
1087 arrowheads indicate juxtavascular microglia that remain on vessels for 6 weeks. Unfilled



1088 arrowhead indicates a juxtavascular microglia that changes position, but remains on the  
1089 vasculature, over 6 weeks. All error bars represent  $\pm$  SEM.

1090

1091 **Figure 6: Juxtavascular microglia contact the cortical vasculature in areas lacking full**

1092 **astrocytic endfoot coverage. A-C.** Representative single optical plane images and 3D

1093 rendering (**Aiv-Civ**; see also Movies 8-10) of juxtavascular microglia (green, EGFP) and blood

1094 vessels (magenta, anti-PDGFR $\beta$ ) in areas void of astrocytic endfoot labeling (gray, anti-AQP4)

1095 in the frontal cortex at P5 (**A**), P7 (**B**) P28 (**C**). Filled arrowheads denote vascular areas that lack

1096 astrocyte endfeet where juxtavascular microglia are contacting the vessel. Scale bars= 10 $\mu$ m.

1097 **D.** Left Y axis, gray bars: quantification of the percent of blood vessels covered by astrocyte

1098 endfeet over development in the frontal cortex. One-way ANOVA with Dunnett's post hoc;

1099 comparison to P $\geq$ 21, n=3 littermates per developmental time point, \*\*\*p<.001, \*\*\*\*p<.0001.

1100 Right Y axis, black line: the percent of the total microglia population that are juxtavascular over

1101 development in the frontal cortex (data are the same as presented in Fig. 1G). One-way ANOVA

1102 with Dunnett's post hoc; comparison to P $\geq$ 21, n=4 littermates per developmental time point,

1103 +++++p<.0001. **E.** Quantification of the percent of juxtavascular microglia contacting vessels

1104 only, vessels and astrocyte endfeet (representative images in A-C), and astrocyte endfeet only

1105 from 3D rendered images. **F-I.** Representative expansion microscopy (ExM, **F-G**) and structured

1106 illumination microscopy (SIM, **H-I**) images of juxtavascular microglia (green, EGFP), in vascular

1107 areas lacking anti-AQP4 (gray) astrocytic endfoot labeling (filled arrowheads) in the P5 (**F, H**)

1108 and P28 (**G, I**) frontal cortex. Scale bars= 10 $\mu$ m. All error bars represent  $\pm$  SEM.

1109

1110 **Figure 7: Ultrastructural analysis by EM reveals that juxtavascular microglia directly**

1111 **contact the basal lamina of the vasculature**

1112 **A-B.** Electron microscopy (EM) of juxtavascular microglia (green pseudocoloring) contacting the

1113 basal lamina (purple line) of a blood vessel in an area void of astrocyte endfeet (blue

1114 pseudocoloring) in the P5 (**A**, left column) and P56 (**B**, right column) frontal cortex. Pink  
1115 pseudocoloring denotes a pericyte. Asterisks denote microglia nuclei. Scale bar= 5µm. The  
1116 black box denotes the magnified inset in the bottom right corner where microglia (green  
1117 pseudocoloring) directly contact the basal lamina (unlabeled in the inset) and only partially  
1118 contacts the astrocyte endfoot (blue pseudocoloring). Scale bar= 1µm. **C**. 3D reconstruction of  
1119 serial EM of P5 juxtavascular microglia in **Aiii (Ci)** and P56 P56 juxtavascular microglia in **Biii**  
1120 (**Cii**) (see also Movies 11 and 12).

1121  
1122 **Movie 1: 3D rendering of juxtavascular microglia in the early postnatal frontal cortex.** 3D  
1123 reconstruction and surface rendering of juxtavascular microglia (green, EGFP) associated with  
1124 blood vessels (magenta, anti-PECAM) in the P5 frontal cortex. Yellow denotes contact area  
1125 between microglia and blood vessels.

1126  
1127 **Movie 2: 3D rendering of juxtavascular microglia in the P28 frontal cortex.** 3D  
1128 reconstruction and surface rendering of juxtavascular microglia (green, EGFP) associated with  
1129 blood vessels (magenta, anti-PECAM) in the P28 frontal cortex. Yellow denotes contact area  
1130 between microglia and blood vessels.

1131  
1132 **Movie 3: Juxtavascular microglial migration in the early postnatal somatosensory cortex.**  
1133 Representative live imaging of juxtavascular microglia (green, EGFP) migrating on vessels  
1134 (magenta; dextran) in the P7 somatosensory. *Cx3cr1<sup>EGFP/+</sup>* mice received a retro-orbital injection  
1135 of Texas red labeled dextran to label the vasculature 10 minutes prior to euthanasia. Coronal  
1136 somatosensory cortices were imaged every 5 minutes over 6 hours immediately following slice  
1137 preparation. Asterisk in still image denotes the microglia that was tracked for quantification.

1138

1139 **Movie 4: Juxtavascular microglial migration in the early postnatal somatosensory cortex.**

1140 A second representative live imaging of juxtavascular microglia (green, EGFP) migrating on  
1141 vessels (magenta; dextran) in the P7 somatosensory. *Cx3cr1*<sup>EGFP/+</sup> mice received a retro-orbital  
1142 injection of Texas red labeled dextran to label the vasculature 10 minutes prior to euthanasia.  
1143 Coronal somatosensory cortices were imaged every 5 minutes over 6 hours immediately  
1144 following slice preparation. Asterisk in still image denotes the microglia that was tracked for  
1145 quantification.

1146

1147 **Movie 5: Juxtavascular microglial migration in the early postnatal somatosensory cortex.**

1148 A third representative live imaging of juxtavascular microglia (green, EGFP) migrating on  
1149 vessels (magenta; dextran) in the P7 somatosensory. *Cx3cr1*<sup>EGFP/+</sup> mice received a retro-orbital  
1150 injection of Texas red labeled dextran to label the vasculature 10 minutes prior to euthanasia.  
1151 Coronal somatosensory cortices were imaged every 5 minutes over 6 hours immediately  
1152 following slice preparation. Asterisk in still image denotes the microglia that was tracked for  
1153 quantification.

1154 .

1155 **Movie 6: Juxtavascular microglial migration in the adult somatosensory cortex.**

1156 Representative live imaging of juxtavascular microglia (green, EGFP) stationary on vessels  
1157 (magenta; dextran) in the P<sub>≥</sub>120 somatosensory cortex. *Cx3cr1*<sup>EGFP/+</sup> mice received a retro-  
1158 orbital injection of Texas red labeled dextran to label the vasculature 10 minutes prior to  
1159 euthanasia. Coronal somatosensory cortices were imaged every 5 minutes over 6 hours  
1160 immediately following slice preparation. Asterisk in still image denotes the microglia that was  
1161 tracked for quantification.

1162

1163

1164 **Movie 7: 2-photon *in vivo* live imaging of juxtavascular microglia in the adult cortex.**

1165 Representative 2-photon *in vivo* live imaging of juxtavascular microglia (green, EGFP) stationary  
1166 on blood vessels (magenta, dextran) over 2 hours *in vivo* in the adult cortex. *Cx3cr1*<sup>EGFP/+</sup> mice  
1167 received a retro-orbital injection of Texas Red-labeled dextran to visualize the vasculature 10  
1168 min prior to each imaging session. EGFP+ juxtavascular microglia were then imaged every 5  
1169 minutes for 2 hours.

1170

1171 **Movie 8: Juxtavascular microglia contact the cortical vasculature in areas lacking full**

1172 **astrocytic endfoot coverage in the P5 frontal cortex.** 3D reconstruction and surface  
1173 rendering of juxtavascular microglia (green, EGFP) contacting blood vessels (magenta, anti-  
1174 PDGFR $\beta$ ) in areas void of astrocytic endfoot labeling (gray, anti-AQP4) in the frontal cortex at  
1175 P5.

1176

1177 **Movie 9: Juxtavascular microglia contact the cortical vasculature in areas lacking full**

1178 **astrocytic endfoot coverage in the P7 frontal cortex.** 3D reconstruction and surface  
1179 rendering of juxtavascular microglia (green, EGFP) contacting blood vessels (magenta, anti-  
1180 PDGFR $\beta$ ) in areas void of astrocytic endfoot labeling (gray, anti-AQP4) in the frontal cortex at  
1181 P7.

1182

1183 **Movie 10: Juxtavascular microglia contact the cortical vasculature in areas lacking full**

1184 **astrocytic endfoot coverage in the P28 frontal cortex.** 3D reconstruction and surface  
1185 rendering of juxtavascular microglia (green, EGFP) contacting blood vessels (magenta, anti-  
1186 PDGFR $\beta$ ) in areas void of astrocytic endfoot labeling (gray, anti-AQP4) in the frontal cortex at  
1187 P28.

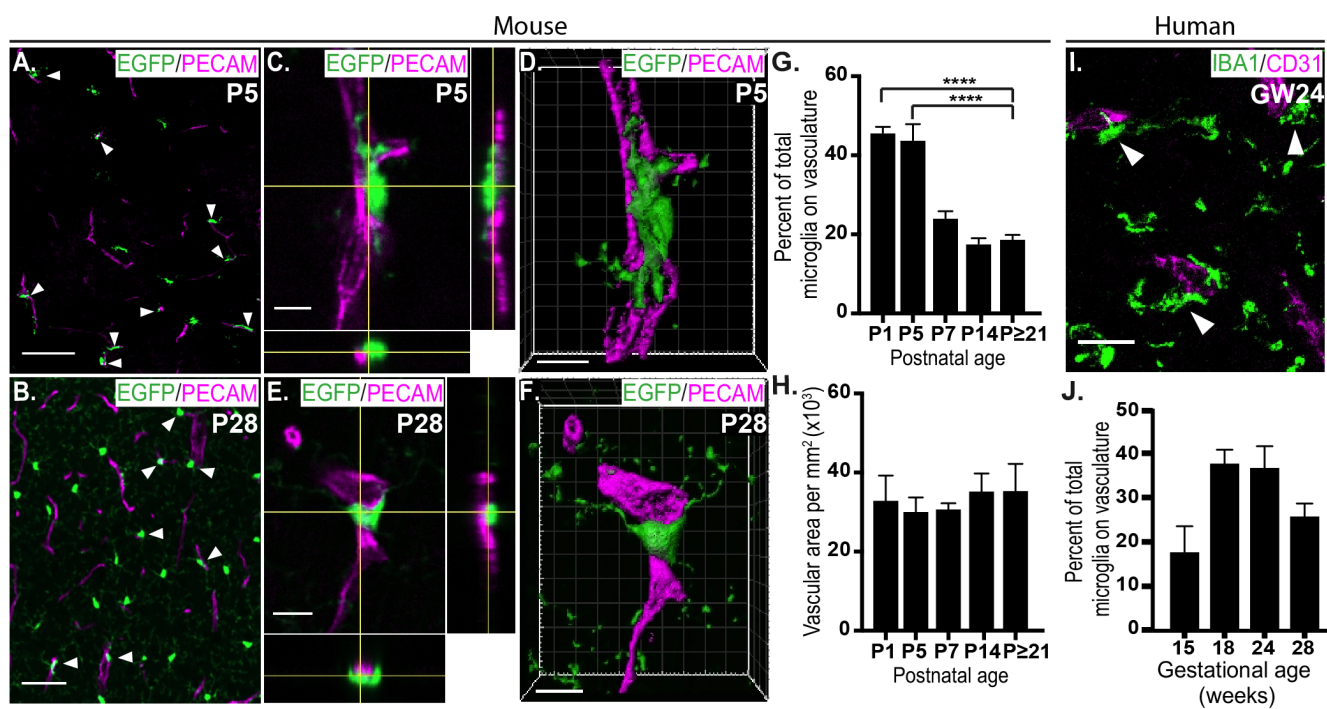
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1189 **Movie 11: Serial EM 3D reconstruction of juxtavascular microglia in the early postnatal**  
1190 **cortex.** 3D reconstruction of serial electron microscopy (EM) of juxtavascular microglia (green)  
1191 contacting the basal lamina (red) of a blood vessel in an area void of astrocyte endfeet (blue) in  
1192 the P5 frontal cortex.

1193  
1194 **Movie 12: Serial EM 3D reconstruction of juxtavascular microglia in the P56 cortex.** 3D  
1195 reconstruction of serial electron microscopy (EM) of juxtavascular microglia (green) contacting  
1196 the basal lamina (red) of a blood vessel lacking full astrocyte endfoot (blue) coverage in the P56  
1197 frontal cortex.

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1215 **Figure 1**



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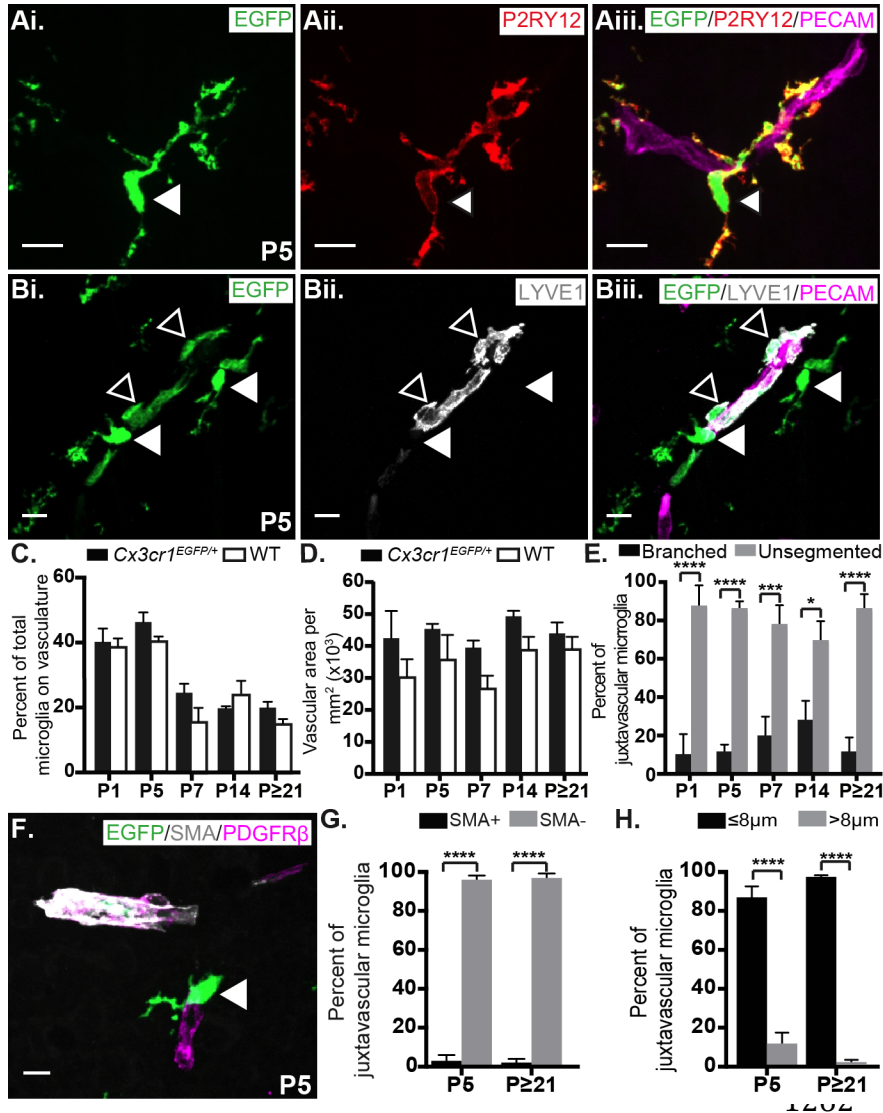
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1230 **Figure 2**



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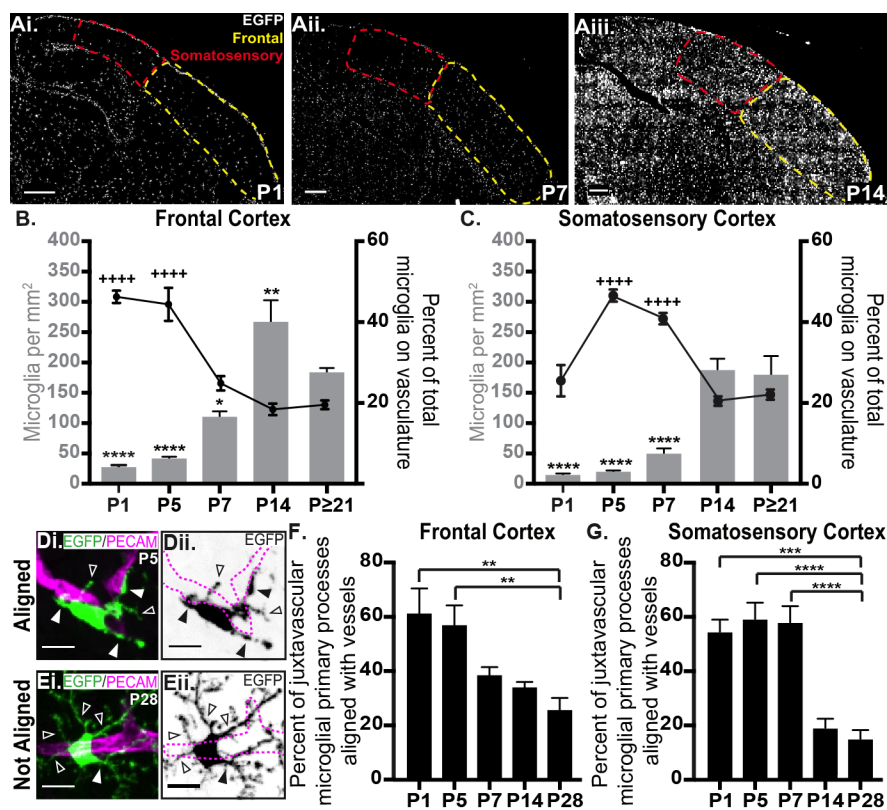
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1271 **Figure 3**



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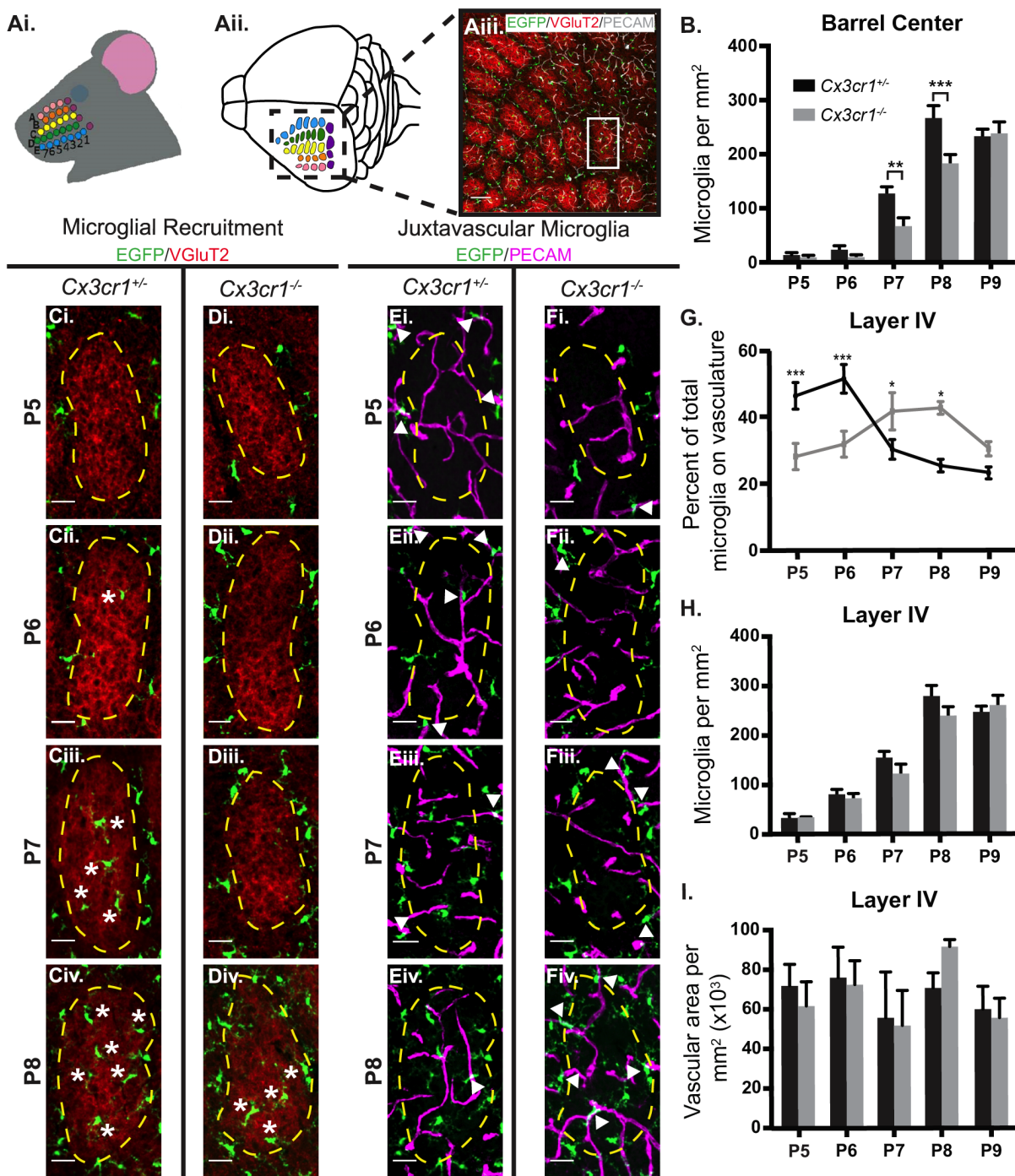
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1303 **Figure 4**



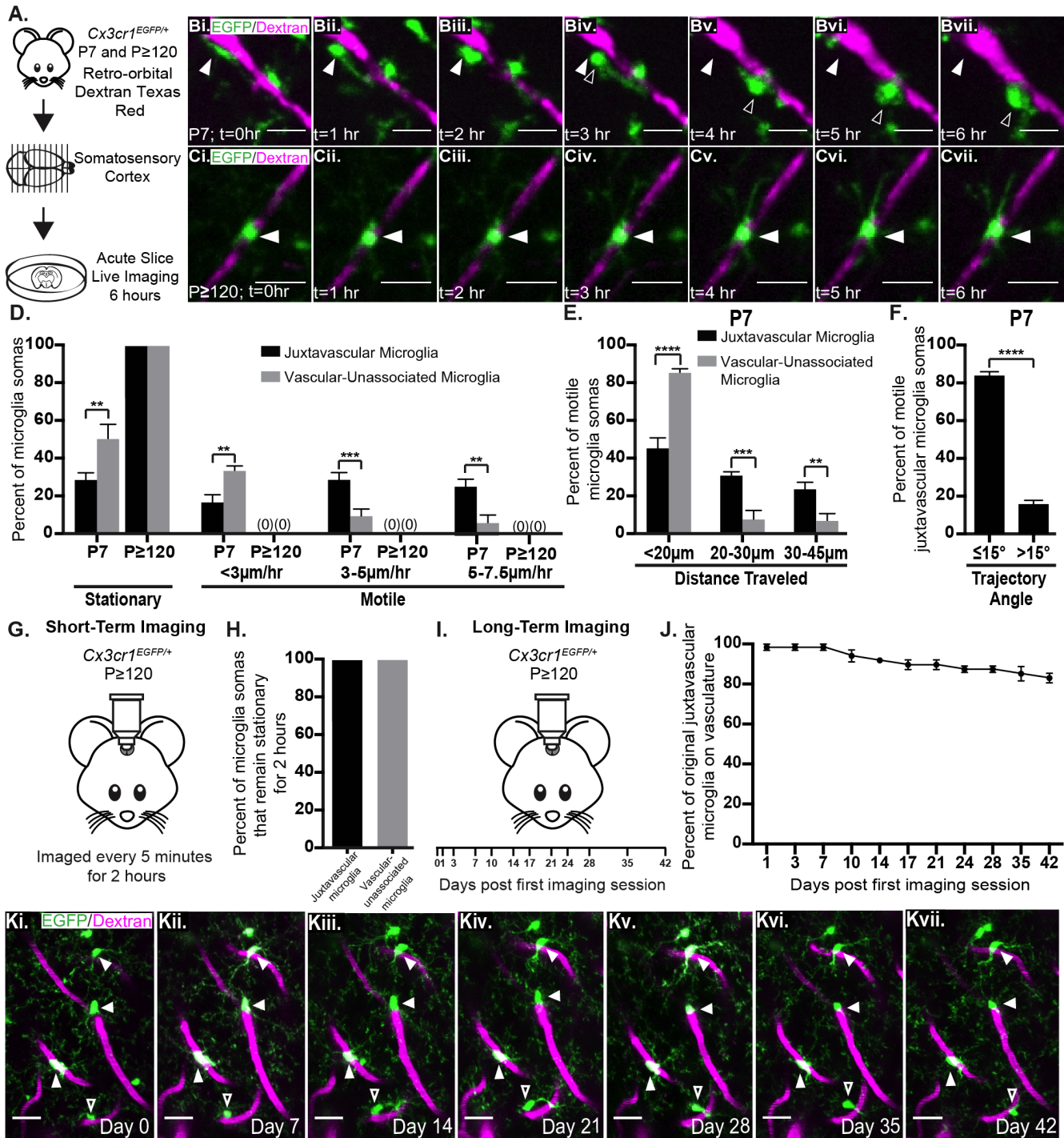
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1308 **Figure 5**



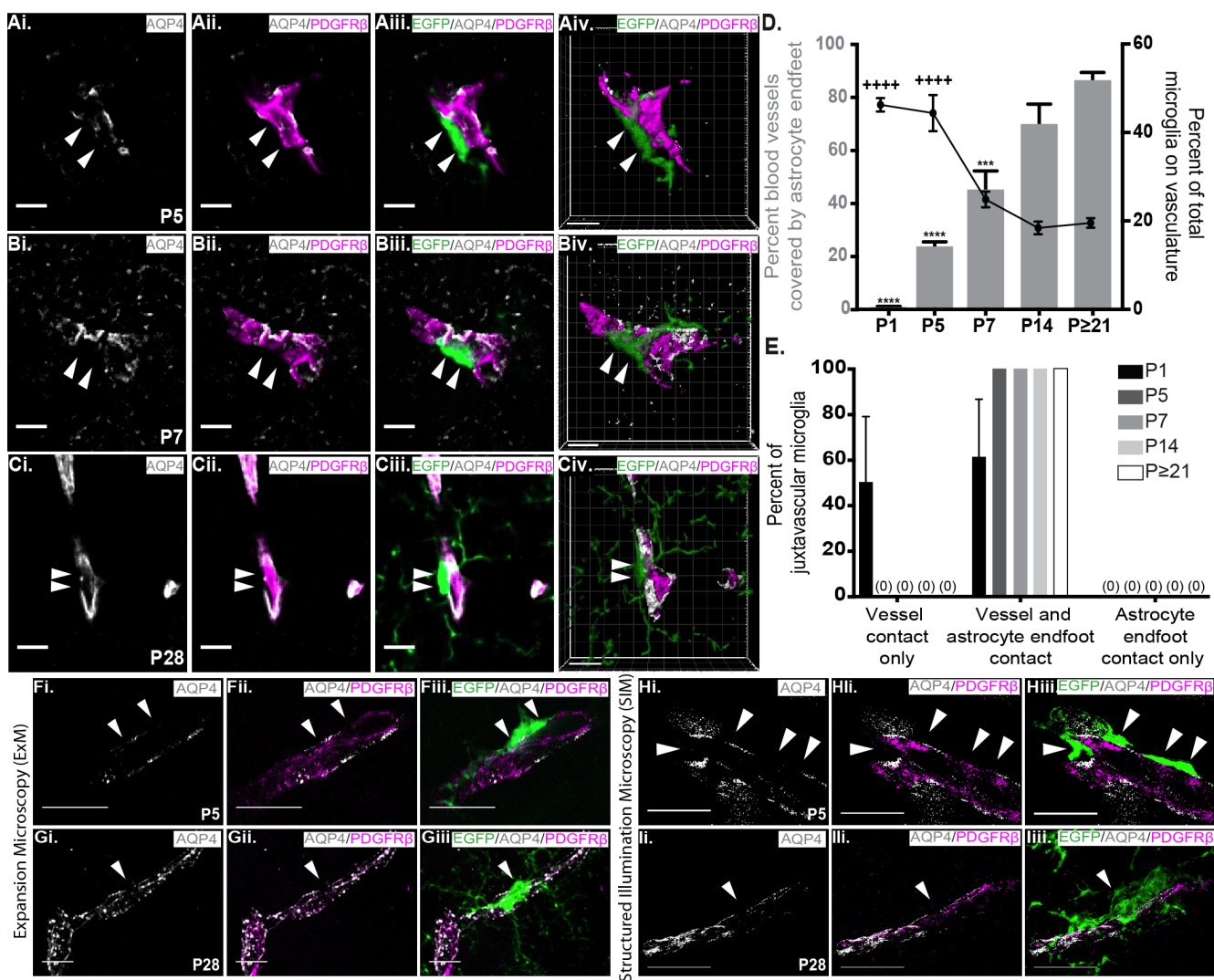
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1313 **Figure 6**



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1323 **Figure 7**

