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The Microglial Fractalkine Receptor is Not Required for Activity-dependent Plasticity in the Mouse Visual System

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Abstract

Microglia have recently been implicated as key regulators of activity-dependent plasticity, where they contribute to the removal of inappropriate or excess synapses. However, the molecular mechanisms that mediate this microglial function are still not well understood. Although multiple studies have implicated fractalkine signaling as a mediator of microglia-neuron communications during synaptic plasticity, it is unclear whether this is a universal signaling mechanism or whether its role is limited to specific brain regions and stages of the lifespan. Here, we examined whether fractalkine signaling mediates microglial contributions to activity-dependent plasticity in the developing and adolescent visual system. Using genetic ablation of fractalkine's cognate receptor, CX₃CR1, and both *ex vivo* characterization and *in vivo* imaging in mice, we examined whether fractalkine signaling is required for microglial dynamics and modulation of synapses, as well as activity-dependent plasticity in the visual system. We did not find a role for fractalkine signaling in mediating microglial properties during visual plasticity. Ablation of CX₃CR1 had no effect on microglial density, distribution, morphology, or motility, in either adolescent or young adult mice across brain regions that include the visual cortex. Ablation of CX₃CR1 also had no effect on baseline synaptic turnover or contact dynamics between microglia and neurons. Finally, we found that fractalkine signaling is not required for either early or late forms of activity-dependent visual system plasticity. These findings suggest that fractalkine is not a universal regulator of synaptic plasticity, but rather has heterogeneous roles in specific brain regions and life stages.

Keywords

CX₃CR1; synaptic plasticity; visual cortex; lateral geniculate nucleus

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INTRODUCTION

The brain reorganizes its circuitry by restructuring individual synapses through a process called synaptic plasticity. The cellular and molecular mechanisms instructing this synaptic rearrangement are of interest not only for understanding the neural basis of behavior, but also for developing treatments for the recovery of function after injury and amelioration of defects in neurodevelopmental and neurodegenerative disorders. While most research on synaptic plasticity has focused on neuron-intrinsic mechanisms, there is growing evidence that non-neuronal cell types, particularly microglia, the immune cells of the brain, contribute to the process of synaptic modification (Bessis et al. 2007; Schafer et al. 2013). Microglia can remove synaptic elements in an activity-dependent manner (Schafer et al. 2012; Tremblay et al. 2010a) and they play a role in early forms of plasticity in the hippocampus and visual system (Paolicelli et al. 2011; Schafer et al. 2012; Zhan et al. 2014). The results of these studies suggest that microglia are responsible for removal of inactive synapses during development. Given the severe cognitive and behavioral consequences that can occur when developmental synapse removal is disrupted, it is critical to understand the precise mechanisms through which microglia contribute to this process.

Although microglial signaling pathways mediating immune response have been explored more comprehensively, mechanisms employed during physiological processes such as synaptic plasticity are relatively unknown. To date, many immune signaling molecules have been implicated in multiple forms of plasticity, suggesting that microglia use similar mechanisms during both physiological and pathological events. For example, canonical immune signaling molecules, such as IL1- β , have been shown to play a role in long-term depression in the hippocampus (Ikegaya et al. 2003). In the visual system, complement signaling and MHC I are necessary for the early developmental process of activity-dependent circuit refinement in the lateral geniculate nucleus, while TNF- α is required for normal ocular dominance plasticity during adolescence (Huh et al. 2000; Kaneko et al. 2008; Schafer et al. 2012). The chemokine fractalkine is known to alter microglial behavior in pathological conditions (Bisht et al. 2016; Cardona et al. 2006; Seo et al. 2016; Wang et al. 2014; Zabel et al. 2016; Zujovic et al. 2000), but could similarly serve physiological roles. Fractalkine is released from neurons in an activity-dependent manner and its only known receptor, CX₃CR1, is expressed specifically in microglia in the brain, providing a potential mechanism for specific signaling between synapses and microglial processes (Bazan et al. 1997; Cardona et al. 2006; Harrison et al. 1998; Imai et al. 1997; Kim et al. 2011). In fact, fractalkine signaling has been implicated in multiple forms of plasticity (Arnoux and Audinat 2015; Audinat and Arnoux 2014; Hoshiko et al. 2012; Maggi et al. 2011; Pagani et al. 2015; Paolicelli et al. 2011; Rogers et al. 2011; Zhan et al. 2014). However, it remains unclear whether microglia use the same signaling molecules to contribute to synapse remodeling throughout the brain and across the lifespan, or whether microglia are heterogeneous in how they implement their physiological functions. Establishing whether fractalkine is a universal mediator of activity-dependent plasticity, or is employed in a selective manner, is necessary to determine the heterogeneity of microglial signaling in physiological conditions.

Here, we show that in the visual system, germ-line knock-out of *Cx3cr1* does not overtly change microglial phenotype. We found no overt defects in cortical microglia density, morphology or dynamics in *Cx3cr1*-null mice. Similarly, we saw no change in the dynamics of dendritic spine turnover or microglia-synapse interactions in adolescent *Cx3cr1*-null mice, suggesting that fractalkine signaling does not affect synaptic remodeling in sensory cortex at this stage of life. Finally, we show that microglial fractalkine signaling is not required for functional plasticity in either early or late periods of visual system development, as there is no defect in lateral geniculate nucleus refinement or ocular dominance plasticity in *Cx3cr1*-null mice. While it has been shown that fractalkine is critical for some forms of plasticity, our findings suggest that fractalkine signaling is not a universal molecular mediator of synaptic plasticity, and that the molecular mechanisms used by microglia during plasticity vary by region and age.

MATERIALS & METHODS

Animals

Experimental protocols were carried out in strict accordance with the University of Rochester Committee on Animal Resources (UCAR) and conformed to the National Institute of Health's "Guide for the Care and Use of Laboratory Animals, 8th Edition, 2011." Experiments characterizing baseline microglial function were carried out at ages representing early development (postnatal day (p) 15), adolescence (p28), or early adulthood (p60). Experiments examining visual cortical plasticity were carried out during the visual critical period for ocular dominance plasticity, between p26 and p34. Monocular deprivations were performed between p26 and p30 by removing the right eye lid margins and suturing the lid shut. Examination of retino-geniculate projections in the lateral geniculate nucleus were carried out during the same time period, as reorganization of these projections is complete by this time and the final organization of eye-specific layers can be assessed. Experiments examining microglial infiltration into thalamocortical axon (TCA) clusters were carried out at p7 and p10 to replicate previously published methods (Hoshiko et al. 2012). Both female and male mice were included in all experiments and all mouse lines were generated on a C57Bl/6 background. C57Bl/6 (Jackson Labs), *Cx3cr1-EGFP* (Jung et al. 2000), *Cx3cr1*-knockout (Taconic Biosciences), and *thy1-YFP* line H (Feng et al. 2000) mouse lines were used and bred together as follows: The *Cx3cr1-EGFP* mouse line was used both to visualize microglia and to achieve manipulation of CX₃CR1. For experiments involving *in vivo* imaging of microglia, because visualization of microglia requires at least one copy of GFP, *Cx3cr1-EGFP* heterozygous mice (*Cx3cr1*^{G/+}) were used as controls. It is important to note that some studies have observed gene dosage effects in *Cx3cr1-EGFP* (Jung et al. 2000; Lee et al. 2010; Rogers et al. 2011). While this finding comes from a small subset of studies conducted under mostly pathological conditions, it is therefore possible that heterozygous mice might not behave the same as wild-type mice. However, given that these experiments cannot be carried out without a fluorescent label, this question will need to be explored using a different approach in the future. Similarly, to assure similar levels of GFP expression and therefore similar visualization of microglia in *Cx3cr1*-null mice as in control mice, *Cx3cr1-EGFP* homozygous mice (*Cx3cr1*^{G/G}) were crossed to *Cx3cr1*-knockout mice (*Cx3cr1*^{-/-}) to generate *Cx3cr1*-null mice with a single

copy of GFP (*Cx3cr1^{G/-}*). *Cx3cr1^{G/G}* mice were included in imaging experiments to assay the potential impact of additional GFP expression on visualization and/or GFP toxicity. We did not observe any differences in the dynamics of microglia expressing different levels of GFP, but we cannot rule out the possibility that GFP overexpression alters microglial behavior. For experiments examining *in vivo* interactions between neurons and microglia, *thy1-YFP* mice were crossed to generate *Cx3cr1^{G/+}/YFP* control mice, as well as both *Cx3cr1^{G/-}/YFP* and *Cx3cr1^{G/G}/YFP* *Cx3cr1*-null mice, again to assay the impact of additional GFP expression. For experiments examining *in vivo* dendritic spine turnover, *YFP*, *Cx3cr1^{G/+}/YFP*, and *Cx3cr1^{G/G}/YFP* mice were used, as microglia were not studied in these experiments.

Histology

Following injection with Euthazol (Virbac), mice were perfused transcardially with 0.1M phosphate buffered saline (PBS) followed by 4% paraformaldehyde (PFA) in 0.1M PBS. Following overnight fixation in 4% PFA at 4°C, brains were cryoprotected with 30% sucrose in 0.2M phosphate buffer (PB). Coronal sections (for Iba1 reactivity) or tangential sections (for 5-HTT reactivity) were cut on a freezing microtome (Microm; Global Medical Instrumentation, Ramsey, MN) at 50 µm thickness into cryoprotectant. Sections were processed free-floating at room temperature (RT), except where noted. Briefly, sections were rinsed in 0.1M PBS, incubated for 20 minutes in a 3% hydrogen peroxidase solution and for 1 hour in blocking buffer. Sections were then incubated in a primary antibody solution (anti-Iba1, 1:1000, Wako, Cat# 019-19741; anti-5-HTT, 1:1000, Calbiochem, Cat# PC177L) for 24 hours at 4°C. Following primary antibody incubation, sections were rinsed with 0.1M PBS and incubated for 4 hours at RT in a secondary antibody solution (Alexa-Fluor 488 or Alexa-Fluor 594, 1:500, Invitrogen, Cat# A-21206, Cat# A-21207). Following a final rinse in 0.1M PBS, sections were mounted on slides and coverslipped with Prolong Gold Antifade Reagent (Molecular Probes, Carlsbad, CA, Cat# P36934).

For examination of microglial density and distribution, primary visual cortex (V1), primary somatosensory cortex (S1), and the CA1 region of the hippocampus were identified using stereotaxic co-ordinates (Paxinos, Elsevier). Iba1 immunoreactivity in each area was imaged using a 10x, 0.30 NA objective on a BX51 Olympus scope (Olympus, Tokyo, Japan) mounted with a Spot Pursuit RT color digital camera (Diagnostic Instruments, Sterling Heights, MI) at uniform exposure settings for each age group. Iba1 positive microglia were identified and counted in ImageJ (National Institutes of Health). The number of cell bodies was then divided by the measured area to generate cell density. To determine the distribution of microglia across the cortical or hippocampal surface a nearest neighbor calculation was carried out for each microglial cell body. This distribution index was calculated as the square of the average nearest neighbor distance multiplied by microglial density on a per image basis. Both density and distribution, individual image values were averaged across all images to determine the value per animal. For examination of microglial morphology and ramification, areas contained entirely within the binocular region of V1 were identified and imaged on a Zeiss LSM 510 confocal microscope (Carl Zeiss, Thornwood, NY) using a 40x, 1.2 NA, water-immersion objective and a z-step of 1µm. Analysis was performed offline in ImageJ on z-projected images of uniform depth. Microglia whose entire process arbor was

contained within the image were analyzed. Microglial soma size was quantified using the ImageJ freeform tool to manually select the cell body and using the measure tool to calculate the area. Microglial arbor size was quantified using the ImageJ trapezoid tool to manually connect the most distal points of the processes of each microglia and using the measure tool to calculate area. Microglial soma and process circularity were quantified by drawing and measuring a line through both the longest axis and the perpendicular shortest axis. Soma and arbor circularity were calculated using the formula $(\text{length} \times \text{width}) / (\text{length} + \text{width})$. Process ramification was assayed by manually selecting individual microglia, including their entire process arbor, and cropping them into a new image. These images were thresholded to create a binarized image, and analyzed using an automated ImageJ Sholl analysis plug-in (kindly provided by the Arnivan Ghosh Lab, UCSD).

For analysis of microglial infiltration into TCA clusters, tangential sections through layer IV of S1 were imaged using a 10x, 0.30 NA objective on a BX51 Olympus scope (Olympus, Tokyo, Japan) mounted with a Spot Pursuit RT color digital camera (Diagnostic Instruments, Sterling Heights, MI) at uniform exposure settings. Intrinsic GFP signal was used to visualize microglia, in accordance with the methods used by Hoshiko and colleagues (Hoshiko et al. 2012). Image analysis was performed offline in ImageJ, where the number of microglial cells inside the TCA cluster (defined by that TCA cluster's borders) and outside the TCA clusters (defined by the borders of all neighboring TCA clusters) were quantified to determine the ratio of microglia inside to outside the TCA clusters. Analysis was performed in 5–6 barrels per animal.

Two-Photon Imaging

In vivo imaging was performed using a custom two-photon laser-scanning microscope. The microscope consists of a Ti:Sapphire laser providing 100 femtosecond pulses at 80MHz at a wavelength of 920nm (Mai Tai, Spectra-Physics) and a modified Fluoview confocal scan head (Olympus). For single channel imaging of GFP, fluorescence was detected using a photomultiplier tube (Hamamatsu) and a 580/180 filter. For dual channel imaging of GFP and YFP, fluorescence was detected using two photomultiplier tubes (Hamamatsu), 491/41 (GFP) or 580/180 (YFP) filters, and a 506 dichroic. GFP and YFP channels were separated offline in ImageJ by subtracting GFP bleedthrough from the YFP channel. Image acquisition was accomplished using a 20x, 0.95 NA lens (Olympus) and Fluoview software. All imaging was carried out between p26 and p30. Mice were anesthetized with fentanyl cocktail [fentanyl (0.05 mg/kg), midazolam (5.0 mg/kg), and dexmedetomidine (0.5 mg/kg), i.p.] and body temperature was maintained at 37°C.

For quantification of microglial motility, an area of the skull was thinned over both V1 and S1 in *Cx3cr1^{G/+}*, *Cx3cr1^{G/-}*, and *Cx3cr1^{G/G}* mice. Z-stack images of microglia were collected at digital zoom 5, every 5 minutes for one hour. Analysis was performed offline in ImageJ and Matlab using custom algorithms. Z-projections of consistent depth were generated for each time interval, concatenated, and corrected for motion artifact. A threshold was applied to all images, and color overlays generated for adjacent sets of time points, resulting in a single image where magenta pixels represent processes present in only the first time point (retraction), green pixels represent processes present in only the second time

point (extension), and white pixels represent processes present in both adjacent time points (stability). A custom Matlab algorithm (Sipe et al. 2016) was used to compare pixels across time points or across overlays and generate a motility index (defined as the sum of all magenta and green pixels divided by all white pixels), a stability index (defined as the proportion of green pixels that became white in a subsequent overlay), and an instability index (defined as the proportion of white pixels which became magenta in a subsequent overlay). For each index, individual microglia values were averaged to generate the value per animal.

Laser ablations were achieved by carrying out a point scan localized at a microglial cell body for 15 s at 780 nm using ~75mW at the sample. The microglial response to laser ablation was performed as described previously (Davalos et al. 2005; Sipe et al. 2016), by quantifying the movement of microglial processes entering an inner radius (X, 1.75x ablation core diameter) centered around the ablation from an outer radius (Y, 3.0x ablation core diameter) over time. Timelapse z-projection images were generated and thresholded to normalize background fluorescence. The total number of pixels in X and Y were measured across time [R_x(t); R_y(t)] and the ablation response was calculated using the equation $R(t) = (R_x(t) - R_x(0)) / R_y(0)$.

For analysis of microglia-dendritic spine contacts, *Cx3cr1^{G/+}/YFP*, *Cx3cr1^{G/-}/YFP*, and *Cx3cr1^{G/G}/YFP* double-transgenic mice were used to visualize both microglia and dendritic spines in the presence or absence of fractalkine signaling. Experiments were carried out in S1, as YFP expression is low in V1 at these ages, and synaptogenesis and synapse dynamics in the two cortical areas are similar (Elston and Fujita 2014). An area of skull over S1 was thinned and z-stack images were collected in both channels at digital zoom 8, every 5 minutes for 2 hours (Xu et al. 2007). Analysis was performed offline in ImageJ. Both channels were corrected for background fluorescence and GFP bleedthrough was subtracted from the YFP channel. All spines contacted by a microglial process were identified, and regions of interest for analysis were defined as dendritic segments extending 5μm in either direction from a contacted spine. All spines in each region of interest were assayed for presence or absence of microglial contact, length of contact, initial spine size, and spine size before, during, and after contact. Spine size was measured in the plane where the spine head was most in focus as the background-subtracted integrated density of a region of interest containing only the spine head. Frequency of contact was assayed as the average per animal of the number of contacted spines divided by the product of the integrated pixel density of dendritic fluorescence and microglial fluorescence.

For analysis of dendritic spine turnover, chronic imaging was performed across a four-day period in *YFP*, *Cx3cr1^{G/+}/YFP*, and *Cx3cr1^{G/G}/YFP* double-transgenic mice. The skull was thinned over S1 and the underlying cortical area was imaged. After the imaging session on day 0, the scalp was sutured closed, the mouse was administered fentanyl reversal agent and allowed to recover at 37°C. Two and four days later, the skull was re-exposed and the same area of S1 was identified based on the blood vessel and dendritic branching pattern and re-imaged. In each imaging session, z-stacks were collected at digital zoom 8 of 5–8 dendritic segments. Analysis was carried out offline in ImageJ. Dendritic spine turnover was quantified by determining the presence and absence of individual dendritic spines along

dendritic spine segments that were visible in all three imaging days. Spines were considered stable if present on all three days, newly stable if present on day two and day four, and transient if only present on day 2.

Electron Microscopy

For electron microscopy (EM) analyses, C57Bl/6 and *Cx3cr1^{G/G}* mice aged p28 were anesthetized with sodium pentobarbital (80 mg/kg, i.p.) and perfused through the aortic arch with 3.5% acrolein. Only mice for which the perfusion was optimal were included in the study. Transverse sections of the brain (50 μ m thick) were cut in sodium phosphate buffer (PBS; 50 mM at pH 7.4) using a vibratome and stored at -20°C in cryoprotectant (30% glycerol and 30% ethylene glycol in PBS) until further processing (Tremblay et al. 2010b).

For immunostaining, sections were immersed in 0.1% sodium borohydride for 30 min at RT, washed in PBS, and processed freely floating following a pre-embedding immunoperoxidase protocol previously described (Tremblay et al. 2010a). Briefly, sections were rinsed in PBS, followed by a 2-hour pre-incubation at RT in a blocking solution of PBS containing 5% normal goat serum and 0.5% gelatin. They were incubated for 48 hour at RT in rabbit anti-Iba1 antibody (1:1,000 in blocking solution; Wako Pure Chemical Industries) and rinsed in PBS. After incubation for 2 hours at RT in goat anti-rabbit IgGs conjugated to biotin (Jackson Immunoresearch) and with streptavidin-horseradish peroxidase (Jackson Immunoresearch) for 1 hour at RT in blocking solution, labeling was revealed with diaminobenzidine (0.05 mg/ml) and hydrogen peroxide (0.03%) in buffer solution (DAB Peroxidase Substrate Kit; Vector Laboratories).

For EM, sections were post-fixed flat in 1% osmium tetroxide and dehydrated in ascending concentrations of ethanol. They were treated with propylene oxide, impregnated in Durcupan (EMS) overnight at RT, mounted between ACLAR embedding films (EMS), and cured at 55°C for 72 hours. Areas of V1, at a level approximating the transverse planes A +0.16 to A +0.72 [63], were excised from the embedding films and re-embedded at the tip of resin blocks. Ultrathin (65–80 nm) sections were cut with an ultramicrotome (Reichert Ultracut E), collected on bare square-mesh grids, stained with lead citrate, and examined with a Hitachi 7650 electron microscope.

In each animal, 80 pictures were randomly taken in layer II of V1, corresponding to a total surface of $1,000\text{ }\mu\text{m}^2$ of neuropil captured per animal. Cellular profiles were identified according to criteria previously defined (Peters et al. 1991; Tremblay et al. 2007; Tremblay et al. 2009; Tremblay et al. 2012). In addition to their Iba1 staining, microglial processes displayed irregular contours with obtuse angles, an electron-dense cytoplasm, numerous large vesicles, occasional multivesicular bodies, frequent phagocytic inclusions, distinctive long stretches of endoplasmic reticulum, and were typically surrounded by pockets of extracellular space (Tremblay et al. 2010a). For quantitative analysis, 50 randomly selected Iba1-positive microglial processes per animal were analyzed in details with ImageJ. For quantification of contacts between microglial processes and synaptic elements, direct juxtapositions between individual processes and dendritic spines, axon terminals, synaptic clefts, or perisynaptic astrocytic processes were counted. For measurement of perimeters of contact between microglial processes and dendritic spines, axon terminals, or perisynaptic

astrocytic processes, all microglial plasma membranes apposing these structural elements were traced with the freehand line tool. A phagocytic index was also compiled by summing up the vacuoles and endosomes containing cellular materials such as membranes, axon terminals with 40-nm synaptic vesicles and dendritic spines with a postsynaptic density in each microglial process analyzed (Tremblay et al. 2010a).

Microcannula Implantation

To assay the impact of temporally-restricted ablation of CX₃CR1 signaling, osmotic minipumps connected to microcannulas were used to achieve intracerebroventricular infusion of CX₃CR1-neutralizing antibody or isotype control, as previously published (Bachstetter et al. 2011; Furuichi et al. 2006). Osmotic minipumps (Alzet; Model#1007D, 0.5uL/hr, 7 days) filled with either CX₃CR1-neutralizing antibody (Torrey Pines Biolabs; 7 µg per day; Cat# TP501) or rabbit IgG isotype control (Sigma; 7 µg per day; Cat# I5006) were implanted subcutaneously between the scapulae of p25 C57Bl/6 mice. These osmotic pumps were connected to a microcannula (Alzet; Cat# 0008851) implanted into the anterior portion of the right lateral ventricle (AP, -0.70; ML, -1.25; DV, -2.15). This area was selected to minimize pathological disruption of left V1, the imaging area of interest. Lack of pathological activation in left V1 was verified via histological processing for MHC I and Iba1, while verification of sufficient spread across hemispheres into the left V1 was confirmed via histology against both anti-CX₃CR1 and rabbit IgG (Supp. Fig. 1). Cannulas which administer continuously for 7 days were chosen and implanted at p25, 3 days prior to monocular deprivation (at p28) to achieve blockade of CX₃CR1 signaling prior to induction of plasticity as well as continued suppression of signaling throughout the 4-day deprivation period.

Intrinsic Optical Signal Imaging

Visual cortical activity was measured using a custom-made intrinsic optical signal imaging setup (Kalatsky and Stryker 2003). To assay the impact of both transgenic and temporal ablation of CX₃CR1 signaling, C57Bl/6, *Cx3cr1^{G/+}*, *Cx3cr1^{G/G}*, C57Bl/6 rabbit IgG-infused, and C57Bl/6 anti- CX₃CR1-infused mice were monocularly deprived between p26 and p30 or left non-deprived as controls. Intrinsic optical signal imaging was carried out 4 days later. The mouse was anesthetized with isoflurane and administered clorprothixene [2mg/kg, i.p.]. If monocularly deprived, the deprived eye was reopened just prior to the imaging session. Both eyes were covered in silicone gel. The skull over visual cortex contralateral to the deprived eye was exposed, covered with 0.5% agar in 0.2M PB, and a coverslip applied and sealed with silicone gel. V1 was illuminated with 700nm light and cortical activity recorded with a CCD camera while either the ipsilateral or contralateral eye was stimulated with a white, horizontal square-wave bar grating on a black background drifting upwards (90°) or downwards (270°) at a frequency of 8°/sec for 6 minutes (30 cm from the eyes). The amplitude of the fast Fourier transform component was averaged across both stimulus directions for each eye individually and compared across eyes offline using a custom Matlab algorithm (Cang et al. 2005). This algorithm computes an ocular dominance index (ODI) using the following equation: $ODI = (\text{average contralateral response} - \text{average ipsilateral response}) / (\text{average contralateral response} + \text{average ipsilateral response})$.

Intraocular Injections

C57Bl/6, *Cx3cr1^{G/+}*, and *Cx3cr1^{G/G}* mice were anesthetized with a mixture of fentanyl (0.05 mg/kg, i.p.), midazolam (5.0 mg/kg, i.p.) and dexmedetomidine (0.5 mg/kg, i.p.). A 33 gauge Hamilton syringe was used to inject anterograde tracer (CtB-AlexaFluor594 or CtB-AlexaFluor647, 0.5% solution in sterile saline, Thermo-Fisher, Cat# C34777, Cat# C34778) into the vitreous fluid. Mice were perfused 24 hours later at p29 and brains harvested and post-fixed in 4% PFA overnight.

LGN Projection Analysis

Coronal brain sections were cut on a freezing microtome. For each animal, 2 sections were analyzed from the middle third of the dorsal lateral geniculate nucleus (dLGN) of each hemisphere (Koch and Ullian 2010; Muir-Robinson et al. 2002). Sections were imaged using a 4x objective on a BX51 Olympus scope (Olympus, Tokyo, Japan) mounted with a Spot Pursuit RT color digital camera (Diagnostic Instruments, Sterling Heights, MI) at uniform exposure settings. Thresholded images were analyzed offline in ImageJ. Ipsilateral area was quantified as the number of pixels present in the fluorescence channel corresponding to the dye injected into the ipsilateral eye normalized by the number of pixels in the entire dLGN. Percent overlap was quantified as the number of pixels overlapping in both channels, measured over a range of thresholds.

RESULTS

Loss of fractalkine signaling does not alter basal microglial density, morphology or dynamics

In order to understand the role of fractalkine signaling in microglia-neuron communication during experience-dependent network remodeling in the visual cortex, we first examined whether germline loss of *Cx3cr1* affects microglial density and morphology within the visual cortex and other brain areas. Fractalkine has been proposed to be involved in the recruitment of microglia into the developing brain (Paolicelli et al. 2011). Because genetic disruption of such a molecule's signaling could result in developmental defects in infiltration of microglia into the brain, we assayed the density of microglia across several brain regions and developmental ages. Using the common microglia marker Iba1 to identify the presence of microglial cell bodies, we found that there was no significant difference in microglia density in V1 at p15, the peak of synaptogenesis, p28, the peak of the visual critical period, or p60, young adulthood, between C57Bl/6, *Cx3cr1^{G/+}*, and *Cx3cr1^{G/G}* mice (Fig. 1A,B). This suggests that fractalkine signaling is not required for the development of normal microglial density in V1. We also found no significant differences in microglia density in S1 or CA1 between the three genotypes, indicating that as previously reported (Hoshiko et al. 2012; Paolicelli et al. 2011), fractalkine signaling deficiency does not result in persistent changes of microglia density within these brain regions (Fig. 1C). Because fractalkine is both a well-known chemoattractant and has also been proposed to regulate the distribution of microglia within brain regions (Arnoux et al. 2013; Hoshiko et al. 2012), we also assayed the distribution of microglia using a nearest neighbor analysis. We found no difference in the distribution of microglia across genotypes in V1 at any age, indicating that microglia infiltrate and distribute throughout V1 properly in the absence of fractalkine signaling (Fig.

1D). These findings were repeated in S1 and CA1 at p28, again indicating that fractalkine signaling deficiency does not result in persistent changes of microglial distribution in these areas (Fig. 1E). In addition to impacts on cell density and distribution, *Cx3cr1*-null mice have been shown to have augmented responses to pathological stimuli (Corona et al. 2010; Liang et al. 2009). Indeed, we confirmed this finding in our model by assaying the microglial response to a laser ablation stimulus in V1. Using *in vivo* two-photon microscopy through a thinned skull, we were able to induce a focal brain injury and subsequently quantify the translocation of microglial processes into proximity of the site of injury over the course of one hour. We found that microglial process infiltration into the site of injury was significantly increased in *Cx3cr1*-null mice compared with control mice, confirming that *Cx3cr1*-null microglia have an altered basal responsiveness to pathological stimuli (Fig. 2A,B; two-way ANOVA; $p = 0.0002$, $F(2,178) = 8.894$). These findings provide insight into the differential functions of fractalkine signaling in maintaining microglial basal state under physiological and pathological contexts.

Fractalkine acts as both a chemoattractant and cell adhesion molecule, and subsequently is capable of altering microglial morphology and dynamics (Cardona et al. 2006; Harrison et al. 1998; Ruitenberg et al. 2008). Microglial morphology is thought to be intimately tied to microglial function, with physiological microglia displaying small cell bodies and large ramified circular arbors. When microglia detect a disturbance they can rapidly hyper-ramify or retract their processes, expand their cell body size and adopt a more elongated shape (Hanisch and Kettenmann 2007; Kettenmann et al. 2011; Ransohoff and Perry 2009). Previously published results demonstrate that *Cx3cr1*-null mice have augmented responses to inflammatory stimuli (Corona et al. 2010; Liang et al. 2009), suggesting that microglia in these mice may exist in an altered basal state. To assay whether CX₃CR1 loss altered microglial state in a manner that impacted microglial morphology, we performed morphological analysis on Iba1-labeled microglia in V1 by measuring the size and circularity of the soma and process arbor (Fig. 3A). In adolescent (p28) mice, we found no significant difference in any measure of morphology (Fig. 3B,C), while in young adult (p60) mice, we observed a difference in process arbor circularity, but this effect was small and apparent only in *Cx3cr1*^{G/+} but not *Cx3cr1*^{G/G} (Fig. 3E,F; one-way ANOVA, $p = 0.0348$, $F(2,10) = 4.785$). In order to more carefully assay microglial process ramification, which may be a more sensitive indicator of microglial morphological changes, we performed a Sholl analysis of branching complexity on Iba1-labelled microglia (Fig. 3A). We found no difference across genotypes in the number of microglial processes at various distances from the soma in either adolescent or young adult mice (Fig. 3D,G), suggesting that CX₃CR1 loss does not impact microglial morphology at baseline in V1.

Microglia are highly motile at baseline (Davalos et al. 2005; Nimmerjahn et al. 2005) and it is likely that this motility is critical to physiological microglial functions which require surveillance and dynamic interactions with other cellular elements. Therefore, we assayed microglial process motility at baseline *in vivo* in the presence or absence of fractalkine signaling. Because visualization of microglia requires expression of at least one copy of GFP, *Cx3cr1*^{G/+} mice were used for comparison to *Cx3cr1*-null mice. Two groups of *Cx3cr1*-null mice, one with a single copy of GFP and one with two copies of GFP, were used to assay for the potential impact of differences in GFP expression on microglial

visualization ($Cx3cr1^{G/-}$ and $Cx3cr1^{G/G}$; see Materials & Methods for details). Using *in vivo* two-photon imaging through a thin skull, we found no difference in process motility, process stability, or process instability between $Cx3cr1^{G/+}$ and $Cx3cr1$ -null mice in either V1 or S1 at p28 (Fig. 4A-G). These results indicate that there is no overt change in microglial baseline function across multiple cortical areas in the absence of fractalkine signaling.

Loss of Fractalkine Signaling Does Not Alter Baseline Microglia-Synapse Dynamics

One of the emerging roles for microglia in the physiological brain is to modulate synapses through secreted signals (Parkhurst et al. 2013) and direct physical contact and phagocytosis (Paolicelli et al. 2011; Schafer et al. 2012; Sipe et al. 2016; Tremblay et al. 2010a). Given this microglia-synapse interaction and fractalkine's dual role as both a secreted and membrane-bound signaling molecule, we assayed whether loss of fractalkine signaling would disrupt the physical interaction between microglia and synaptic elements. Our ultrastructural analysis quantifying direct juxtapositions between microglial processes and synaptic elements at high spatial resolution in C57Bl/6 vs. $Cx3cr1^{G/G}$ mice only revealed subtle differences in adolescent V1 (Fig. 5A,B). For instance, the number of microglial contacts with dendritic spines, synaptic clefts, or perisynaptic astrocytic processes was similar between genotypes, although the number of contacts with axon terminals was significantly decreased in the $Cx3cr1^{G/G}$ mice, within V1 at p28 (Fig. 5C-F; unpaired t-test, $t(4) = 3.546$, $p = 0.0239$). Additionally, the perimeter of microglial contact with dendritic spines and axon terminals was unchanged (Fig. 5G,H), while the perimeter of contact with perisynaptic astrocytic processes was significantly increased in the $Cx3cr1^{G/G}$ mice, as measured in V1 at p28 (Fig. 5I; unpaired t-test, $t(4) = 3.777$, $p = 0.0195$). In line with the previous findings that microglial phagocytosis of amyloid- β is increased in a mouse model of Alzheimer's disease in the absence of CX₃CR1 (Lee et al. 2010), and similarly exacerbated during steady-state conditions and upon stress in the $Cx3cr1^{G/G}$ mice (Miliore et al. 2015), our analysis in developing visual cortex also revealed an increased prevalence of microglial phagocytic inclusions in the $Cx3cr1^{G/G}$ mice V1 at p28 (Fig. 5J; unpaired t-test, $t(4) = 9.127$, $p = 0.0008$).

Because microglia are known to make dynamic contacts with synaptic elements during normal physiology in the absence of experimental manipulation (Tremblay et al. 2010a; Wake et al. 2009), we examined whether disrupting fractalkine signaling would alter baseline microglia-synapse dynamics *in vivo*. To assay microglia-synapse dynamics, we imaged double transgenic mice expressing YFP in a subset of neurons and GFP in microglia *in vivo* every five minutes over an hour in p28 animals (Fig. 6A). This allowed us to characterize elements of the contact as well as the impact of the presence or absence of contact on the dendritic spines. In line with previous research (Tremblay et al. 2010a), we found that in adolescent mice, contacted spines were significantly smaller than spines without contact across genotypes, with no significant effect of genotype on the size of spines that were contacted vs. not contacted (Fig. 6B; two-way ANOVA, main effect of contact $p < 0.0001$, $F(1,24) = 30.44$; genotype $p = 0.1272$, $F(2,24) = 2.250$). Additionally, while small contacted spines grew significantly on microglial contact as compared with large spines, this effect was similar across genotype (Fig. 6C; two-way ANOVA, main effect of spine size $p <$

0.0001, $F(1,24) = 24.17$; genotype $p = 0.4839$, $F(2,24) = 0.7483$). Finally, neither the average duration nor the frequency of microglial contact with dendritic spines were significantly different across genotypes (Fig. 6D,E).

Because we observed increased phagocytosis in *Cx3cr1*-null microglia and microglia have been implicated in synaptic pruning, we wondered whether synaptic dynamics were dependent on fractalkine signaling. Dendritic spines, the postsynaptic sites of the majority of excitatory synapses in the cortex, show remarkable dynamics, with loss and growth of spines apparent in a matter of days (Grutzendler et al. 2002; Trachtenberg et al. 2002). Such synaptic remodeling is thought to reflect intrinsic plasticity within the system as networks rewire (Xu et al. 2009; Yang et al. 2009). To determine whether these intrinsic dynamics of dendritic spines were affected by loss of fractalkine signaling, we used two-photon microscopy to chronically monitor the same dendritic branches *in vivo* through a thin skull over a period of four days in *YFP*, *Cx3cr1^{G/+}/YFP*, and *Cx3cr1^{G/G}/YFP* mice. Dendritic spines were monitored on imaging day 0 and then reimaged two days later at day 2 and day 4 (Fig. 7A). This allowed us to determine the proportion of stable spines (present in all imaging sessions), transient spines (new on day 2 but lost by day 4) and newly stable spines that appeared in day 2, were incorporated into the circuit and were present at day 4. These three categories represent different behaviors that characterize the stability of the dendritic spine networks imaged. We found no difference in any of these categories in *YFP*, *Cx3cr1^{G/+}/YFP*, and *Cx3cr1^{G/G}/YFP* mice (Fig. 7B), indicating that removal of fractalkine signaling does not disrupt baseline spine turnover rate, and that changes in neuronal circuitry as a result of fractalkine removal are not due to a general defect in spine dynamics.

Fractalkine Signaling is Not Required for Early or Late Forms of Visual System Plasticity

Our data thus far suggested that loss of CX₃CR1 had a limited effect on the baseline functions of V1 microglia and synapses. Because fractalkine signaling had been shown to be important for plasticity in the hippocampus (Maggi et al. 2011; Paolicelli et al. 2011; Rogers et al. 2011), we wondered whether, despite the lack of baseline effects of CX₃CR1 loss, fractalkine was also necessary for plasticity in V1, and subsequently whether microglial signaling mechanisms are broadly applicable across different forms of plasticity. Ocular dominance plasticity is a well-characterized model of adolescent experience-dependent plasticity that has yielded understanding of general mechanisms of plasticity that are applicable to other brain regions and functions (Tropea et al. 2009; Wiesel and Hubel 1963a). In this model, closure of one eye during the visual critical period results in alterations in both the structure and function of neuronal connections related to binocular responses in visual cortex (Frenkel and Bear 2004; Gordon and Stryker 1996; Wiesel and Hubel 1963b). Using this model, we assayed whether fractalkine signaling is required for these changes in network function that occur during activity-dependent plasticity. Using intrinsic optical signal imaging to assay functional ocular dominance plasticity in adolescent mice, we found that in adolescence (at ~p32), *C57Bl/6*, *Cx3cr1^{G/+}*, and *Cx3cr1^{G/G}* mice all displayed a strong contralateral bias, indicative of normal visual system circuit development (Fig. 8A,B). In response to a period of 4 days of monocular deprivation, all genotypes exhibited a significant ocular dominance shift (Fig. 8B; two-way ANOVA, main effect of deprivation $p < 0.0001$, $F(1,78) = 181.2$), indicating that normal ocular dominance plasticity

occurs in the absence of fractalkine signaling and suggesting that fractalkine signaling is not required for adolescent activity-dependent plasticity in the visual system.

While we did not find a defect in ocular dominance plasticity using a transgenic ablation model, the germline deletion of *Cx3cr1* could result in compensatory mechanisms allowing visual system plasticity to occur in the absence of CX₃CR1. To address this possibility, we repeated this experiment with temporally-restricted ablation of CX₃CR1 signaling accomplished via administration of a CX₃CR1-neutralizing antibody just prior to and throughout the monocular deprivation period. We found that non-deprived CX₃CR1-ablated and IgG control mice both exhibited a strong contralateral bias indicative of normal circuit development (Fig. 8A,B). In response to a period of 4 days of monocular deprivation, both CX₃CR1-ablated and IgG control mice exhibited a significant ocular dominance shift (Fig. 8B; two-way ANOVA, main effect of deprivation $p < 0.0001$, $F(1,78) = 181.2$), indicating that normal ocular dominance plasticity occurs in the case of temporally-restricted CX₃CR1 ablation as well. These findings further suggest that fractalkine signaling is not required for adolescent activity-dependent plasticity in the visual system.

We have recently demonstrated that microglial processes hyper-ramify after just 12 hours of monocular deprivation (Sipe et al. 2016). This response occurs prior to the period of synapse rearrangement and suggests that microglial process hyper-ramification may instruct circuit rearrangement during ocular dominance plasticity. To determine the role of fractalkine signaling in this process, we again performed Sholl analysis to assay microglial process ramification after 12 hours of monocular deprivation. We found that in line with previous results, microglial processes were significantly hyper-ramified after 12 hours of monocular deprivation, regardless of genotype (Fig. 9A,B; two-way ANOVA, deprivation $p < 0.0001$, $F(5,567) = 197.3$). This suggests that microglia do not use fractalkine signaling during their responses to monocular deprivation.

Given the consistency of our findings across both genetic and temporal ablation of CX₃CR1, our data strongly support the idea that fractalkine does not play a role in adolescent visual system plasticity. However, it is possible that microglia use fractalkine signaling to implement plasticity in an earlier window of development as has been described by others (Hoshiko et al. 2012; Paolicelli et al. 2011). To determine whether microglial CX₃CR1 contributes to early development of microglial functions, we assayed how its loss affected microglial infiltration into somatosensory barrels, a phenomenon which has previously been described to be dependent on CX₃CR1 (Hoshiko et al. 2012). Microglia initially infiltrate TCA clusters of the somatosensory barrel fields at P7. Loss of CX₃CR1 results in a delay in this infiltration, with microglia arriving in barrels by p9 (Hoshiko et al. 2012). We assayed microglial infiltration into TCA clusters in tangential sections of S1 at p7 and p10 using the intrinsic GFP signal as a microglial marker and comparing *Cx3cr1*^{G/G} to *Cx3cr1*^{G/+} controls, as was done previously (Hoshiko et al. 2012). Consistent with published results, we found a significant defect in the number of microglia inside TCA clusters in *Cx3cr1*-null mice at p7, while there was no significant difference between genotypes at p10 (Fig. 10A,B; two-way ANOVA; genotype $p = 0.002$, $F(1,19) = 12.78$). These findings confirm that loss of fractalkine signaling results in a transient defect of microglial infiltration into TCA clusters during barrel field development.

Because we were able to show deficits in microglial behavior with loss of CX₃CR1 during early development as had been previously described, and because fractalkine has been implicated in synapse remodeling earlier in development within the hippocampus (Paolicelli et al. 2011), we wondered whether CX₃CR1 played a role in earlier visual system development. We assayed this by analyzing retinogeniculate remodeling in the lateral geniculate nucleus (LGN), an early developmental process that is dependent on microglial phagocytosis of synapses (Schafer et al. 2012). Developmental remodeling of retinogeniculate projections in the LGN is a competitive process that occurs in two parts: the consolidation of ipsilateral projections, and the exclusion of contralateral projections from the ipsilateral region (Koch et al. 2011; Koch and Ullian 2010). We assayed the size of the ipsilateral region as well as the amount of overlap between ipsilateral and contralateral regions in C57Bl/6, *Cx3cr1*^{G/+}, and *Cx3cr1*^{G/G} mice (Fig. 11A). We found there was no difference in either the area occupied by ipsilateral retinogeniculate projections (Fig. 11B) or the percent overlap between ipsilateral and contralateral projections (Fig. 11C) across genotypes. These results indicate that the developmental organization of projections in the LGN proceeds normally in the absence of fractalkine and therefore fractalkine is not required for this early form of visual system plasticity.

DISCUSSION

Fractalkine signaling has been suggested to be a critical form of neuron-microglia communication during synaptic plasticity in several brain regions (Bachstetter et al. 2011; Hoshiko et al. 2012; Maggi et al. 2011; Paolicelli et al. 2011; Rogers et al. 2011; Zhan et al. 2014), but the question remains of whether this signaling pathway is critical to synaptic remodeling throughout the brain. Here we examined whether the fractalkine signaling pathway is a necessary component of microglia-synapse crosstalk during activity-dependent plasticity in the visual system. Via genetic ablation of the fractalkine receptor, we tested the necessity of fractalkine signaling in basal microglial function and microglia-neuron interactions, as well as in the process of experience-dependent visual system plasticity. While we find that loss of fractalkine signaling altered microglial responses to injury, and delayed microglial infiltration of barrels in somatosensory cortex during early development, we find no evidence that fractalkine signaling is required for microglial homeostatic behavior, such as establishment of microglial density, morphology, motility, and contact with neuronal elements. Additionally, we find that fractalkine signaling is not required for either postnatal or adolescent experience-dependent plasticity in the visual system. These findings suggest that fractalkine is not a universal regulator of developmental plasticity, but rather has limited roles in specific areas and ages.

Fractalkine signaling and homeostatic microglial behavior

Fractalkine is a chemokine, capable of instigating changes in microglial process motility and morphology by inducing structural alterations of the actin cytoskeleton via CX₃CR1-mediated stimulation of the PI3K and MAPK downstream signaling pathways (Cambien et al. 2001; Harrison et al. 1998; Kansra et al. 2001; Maciejewski-Lenoir et al. 1999). It is therefore possible that genetic ablation of *Cx3cr1* could lead to changes in microglial baseline morphology and motility, which could impact their ability to carry out basic

homeostatic functions. Indeed changes in microglia density and distribution have been reported in developing hippocampus and somatosensory cortex (Hoshiko et al. 2012; Paolicelli et al. 2011). While we confirmed changes in microglia distribution in somatosensory cortex in early development, we did not find similar changes within visual cortex across multiple ages; the first indication that the function of CX₃CR1 in physiology may differ across brain regions. Changes in microglial morphology in the absence of CX₃CR1 have also been described. Pagani et al. have found that in very young mice (p8) lacking CX₃CR1, microglia in the hippocampal CA1 region have fewer processes but show no difference in process motility. The authors also suggest that only a small subset of microglia may be affected (Pagani et al. 2015). Other papers investigating the role of CX₃CR1 in the microglial response to stress found increased cell body and arbor area in absence of CX₃CR1 in adult mice in the CA1 region or dentate gyrus of the hippocampus (Miliot et al. 2015; Reshef et al. 2014). However, another study of the microglial response to stress in a different paradigm found no differences in microglia morphology in the dentate gyrus of *Cx3cr1*-null mice, as measured by Iba1 density, process length, number of branching points, number of terminal end points, and Sholl intersections, suggesting further variability in microglial morphological response to the loss of CX₃CR1 (Hellwig et al. 2015). We found no difference across genotypes in microglial morphology in V1 during development or adulthood, and no difference in process motility across cortical areas during development. This result could be explained by heterogeneity of microglial populations, resulting in a spectrum of microglial phenotypes. The majority of knowledge on microglia morphology and motility has been gathered from studies conducted under pathological conditions. However, a small subset of papers has also demonstrated that in the healthy brain, microglia exhibit regional heterogeneity of microglial morphological attributes. These papers found that microglia are sensitive to their microenvironment, resulting in regional differences in density, distribution, and morphology, and suggest that these differences may depend on varying levels of synaptic activity across brain regions (Arnoux et al. 2013; Lawson et al. 1990; Vela et al. 1995). Additionally, microglia may exhibit region-specific heterogeneity of immunoregulatory molecule expression in the intact brain (de Haas et al. 2008). A very recent study that was the first to perform a genome-wide analysis of microglia across multiple brain regions found that microglia exhibit region-dependent heterogeneity of transcriptional identities and suggests that this could explain region-specific homeostatic functions (Grabert et al. 2016). In line with this region-dependent model of microglial physiological function, our findings suggest that fractalkine signaling is not universally required for the ability of microglia to perform baseline homeostatic functions across all ages and brain regions, and underscores the variability in the role of fractalkine signaling across physiological processes, developmental time points, and brain areas.

Fractalkine signaling and microglia-neuron interactions

The discovery that microglia are capable of modulating synapses has opened an entirely new line of research for investigating how synapses are removed during periods of plasticity. Fractalkine is an ideal molecular mediator of this role as it is released in an activity-dependent manner by neurons, is capable of specific signaling between neurons and microglia, and can elicit the process motility necessary to recruit microglia to synapses in need of removal (Bazan et al. 1997; Cardona et al. 2006; Combadiere et al. 1998; Harrison

et al. 1998; Hundhausen et al. 2003; Imai et al. 1997; Jung et al. 2000; Kim et al. 2011; Rossi et al. 1998; Ruitenberget al. 2008; Tsou et al. 2001). In fact, loss of CX₃CR1 has been demonstrated to result in defective synapse elimination early in development within the hippocampus (Paolicelli et al. 2011). If fractalkine is indeed playing a role in synaptic modification, given the dual expression of fractalkine in both a soluble and membrane-bound form, fractalkine could mediate microglia-neuron interactions via secreted signal or upon contact. To examine this possibility, we used EM and *in vivo* dynamic analysis to look at structural contacts between microglia and neurons. We saw very limited changes in the contacts between microglial processes and synaptic elements at the EM level, and no change in microglia-dendritic spines contacts using *in vivo* two-photon microscopy. Consistent with previous findings in wild-type animals (Tremblay et al. 2010a), microglia that lack CX₃CR1 still specifically target small spines, and their contact induces a transient increase in spine size. Contact duration and frequency do not differ in the presence and absence of fractalkine signaling. We did, however, find increased basal phagocytosis in *Cx3cr1*-null animals, as has recently been described in the adult hippocampus (Miliore et al. 2015). Because microglia have been implicated in synaptic phagocytosis, we wondered whether this increased phagocytic capacity altered the removal of synapses at baseline in sensory cortex. We imaged dendritic spines *in vivo* over a period of 4 days and found that rates of spine loss are unaltered in the absence of CX₃CR1, suggesting that CX₃CR1 does not play a role in regulating dendritic spine turnover. Because microglia have also been shown to affect the maturity and strength of existing synapses (Hoshiko et al. 2012; Paolicelli et al. 2011), characteristics that are tied to dendritic spine turnover, we examined the proportion of new spines that were still present on the last day of imaging, and therefore were likely to be stabilized, mature and get incorporated into the network. We found no difference in this population of spines across genotypes. The variability in impact of *Cx3cr1* deletion on dendritic spine modulation in cortex vs. hippocampus could be explained by known differences in the rate of spine turnover in CA1 versus sensory regions of neocortex. It was recently discovered that the CA1 region has a much higher rate of spine turnover than sensory regions in the neocortex (Attardo 2016). As noted by the authors, this high rate of spine turnover could underlie the transient nature of hippocampal-dependent memories and could provide an explanation for the differences in signaling molecules used to regulate dendritic spine stability in the cortex and hippocampus. Taken together, our findings suggest that fractalkine signaling has a limited effect on microglia-synapse structural interactions and synaptic dynamics in sensory cortex during adolescence.

Fractalkine signaling and synaptic plasticity

While it is now established that microglia play physiological roles in addition to their traditional immune functions, it is still unknown to what extent microglia use the same molecular tools in each situation. There is evidence that immune signaling molecules impact the physiological process of plasticity, and specifically visual system plasticity. A number of immune signaling molecules have been implicated in proper developmental pruning of retinogeniculate projections in the LGN. Disruptions of MHC I, neuronal pentraxins (NP1/2), and components of the complement signaling cascade (C1q and C3) have all been demonstrated to cause defects in eye-specific layer formation, and additionally defects in synapse maturation in the cases of MHC I and the complement cascade (Datwani et al.

2009; Fourgeaud et al. 2010; Huh et al. 2000; Koch and Ullian 2010; Schafer et al. 2012; Stevens et al. 2007). The findings presented here suggest that fractalkine could be used in this context as well. Recently there has been increasing evidence for the role of fractalkine in physiological processes such as hippocampal long-term potentiation, neurogenesis, responses to stress, and depression (Bachstetter et al. 2011; Hellwig et al. 2015; Maggi et al. 2011; Milior et al. 2015; Rogers et al. 2011). While there is increasing evidence that fractalkine signaling plays a role in hippocampal dependent forms of plasticity, there is also mounting evidence that fractalkine signaling is not implicated in other regions such as the olfactory bulb, where genetic and pharmacological ablation of CX₃CR1 signaling failed to impact olfactory bulb-related memory and neurogenesis (Reshef et al. 2014). In line with these findings, we demonstrate that fractalkine signaling is not required for visual system plasticity in either early postnatal or later adolescent periods, despite the fact that microglia have been shown to be critical in this process via mechanisms that involve complement and purinergic signaling, respectively (Schafer et al. 2012; Sipe et al. 2016). In the case of ocular dominance plasticity, we show that our findings are not due to developmental compensation for the loss of CX₃CR1 as similar results were obtained with temporally controlled pharmacological methods to remove CX₃CR1 signaling. It is interesting that we see no effect of fractalkine loss on retinogeniculate remodeling, as we confirm that CX₃CR1 is important for microglial function in somatosensory cortex around the same time during development. One factor that could explain the heterogeneity of fractalkine signaling's effects on plasticity is the expression of the fractalkine ligand. Fractalkine is expressed very highly in the hippocampus, particularly in the CA1 region (Kim et al. 2011; Taroazzo et al. 2003), but its expression may be lower in the cortex. Some research has shown that cortical expression of fractalkine is limited to specific areas and cortical layers, such as layer II (Kim et al. 2011), although this study was done using a transgenic reporter line and may not accurately reflect endogenous expression. Others have found much stronger expression in cortex, including all layers except layer I (Taroazzo et al. 2003). This differential expression of the fractalkine ligand could validate a very region-specific role of fractalkine and should be examined carefully in the future, especially in the context of this study where imaging was limited to layers I-II. Taken together, these findings demonstrate that while microglia may recycle their immune signaling repertoire in physiological functions, individual molecules may be employed selectively in some, but not all, functions. Broadly, this indicates that manipulating an individual signaling pathway in microglia is unlikely to achieve a global manipulation of microglia that is desirable for potential therapeutic interventions (Seo et al. 2016; Wieghofer et al. 2015; Zabel et al. 2016). More specifically, this suggests that the role of fractalkine signaling in plasticity is dependent on both the developmental period and the developmental process, underlining the importance of environmental context in microglial function.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Main Points

- Fractalkine signaling is not necessary for microglial roles in synaptic plasticity in the visual system during both early and late development.
- Fractalkine signaling is not a universal regulator of synaptic plasticity.

Figure 1.

Microglial density is unaltered in *Cx3cr1*-null mice.

A, Representative images showing Iba1+ microglia in binocular primary visual cortex of C57Bl/6, *Cx3cr1*^{G/+}, and *Cx3cr1*^{G/G} mice at p28. Genotype names have been abbreviated in figure labels due to space restrictions. Scale bar = 100μm. **B**, There is no significant difference in microglia density in binocular primary visual cortex across genotypes at p15, p28, or p60 (n = 4–7 mice per genotype, one-way ANOVAs; p15: $p = 0.3752$, $F(2,12) = 1.065$; p28: $p = 0.5936$, $F(2,14) = 0.542$; p60: $p = 0.3245$, $F(2,11) = 1.249$). **C**, There is no significant difference in microglia density across genotypes in S1 or the CA1 region of the hippocampus at p28 (n = 4–5 mice per genotype, one-way ANOVAs; S1: $p = 0.1848$, $F(2,12) = 1.950$; CA1: $p = 0.7430$, $F(2,10) = 0.306$). **D–F**, There is no significant difference in microglia distribution measured using a nearest neighbor analysis across age or brain region (age: n = 4–7 mice per genotype, one-way ANOVAs; p15: $p = 0.0765$, $F(2,10) = 3.361$; p28: $p = 0.1476$, $F(2,12) = 2.254$; p60: $p = 0.1869$, $F(2,11) = 1.961$; region: n = 4–5 mice per genotype, one-way ANOVAs; S1: $p = 0.5226$, $F(2,12) = 0.6854$; CA1: $p = 0.7145$, $F(2,10) = 0.3477$). Graphs show mean ± SEM.

Figure 2.

Microglial response to laser ablation is amplified in *Cx3cr1*-null mice.

A, Representative images of microglial response to laser ablation insult in binocular primary visual cortex of *Cx3cr1*^{G/+}, *Cx3cr1*^{G/-}, or *Cx3cr1*^{G/G} mice over 1 hour. Scale bar= 20μm. **B**, Microglial process infiltration into the area proximal to the ablation core (Rx) from an outer core (Ry) were measured across time and the process response was calculated using the equation $R(t)=(R_x(t)-R_x(0))/R_y(0)$. *Cx3cr1*^{G/-} and *Cx3cr1*^{G/G} null mice have significantly elevated process response to pathological injury (n = 4–7 mice per genotype, two-way ANOVA; interaction $p = 0.1086$, $F(22,178) = 1.423$; genotype $p = 0.0002$, $F(2,178) = 8.894$; time $p < 0.0001$, $F(11,178) = 12.16$). Graphs show mean $\pm$ SEM.

Figure 3.

Microglial morphology is unaltered by loss of CX₃CR1.

Morphological analysis of microglia in V1 of p28 (**A-D**) and p60 (**E-G**) mice. **A**, Representative images of soma morphology, process morphology, and Sholl analysis on Iba1+ microglia. Scale bar= 20 μm . **B,C**, There is no significant difference in either soma area or circularity (**B**) or process area or circularity (**C**) across genotypes at p28 (n = 5 mice per genotype, one-way ANOVAs; **B**: $p = 0.1697$, $F(2,12) = 2.064$; $p = 0.0648$, $F(2,12) = 3.467$; **C**: $p = 0.6029$, $F(2,12) = 0.5280$). **E,F**, There is no significant difference in soma area,

soma circularity (**E**) or process arbor area (**F**) across genotypes at p60 (n = 4–5 mice per genotype, one-way ANOVAs; **E**: $p = 0.0995$, $F(2,10) = 2.932$; $p = 0.2950$, $F(2,10) = 1.383$; **F**: $p = 0.2372$, $F(2,10) = 1.667$). At p60 there is a slight increase in process arbor circularity (**F**) in *Cx3cr1*^{G/+} mice as compared to controls. (**F**: n = 4–5 mice per genotype, one-way ANOVA, $p = 0.0348$, $F(2,10) = 4.785$, Bonferroni post-hoc test, $p < 0.05$). **D,G**, Sholl analysis of microglia process ramification revealed no difference in microglia process number across genotypes in V1 of either p28 (**D**) or p60 (**G**) mice (**D**: n = 5 mice per genotype, two-way ANOVA; interaction $p = 0.9999$, $F(48, 300) = 0.3026$; genotype $p = 0.0769$, $F(2,300) = 2.587$; **G**: n = 4–5 mice per genotype, two-way ANOVA; interaction $p = 0.999$, $F(48,300) = 0.3388$; genotype $p = 0.0885$, $F(2,300) = 2.444$). Graphs show mean $\pm$ SEM.

Figure 4.

Microglial motility is unaltered by the loss of CX₃CR1.

A, Schematic representation of motility analysis. Images of *Cx3cr1*^{G/+}, *Cx3cr1*^{G/-}, or *Cx3cr1*^{G/G} microglia were collected every 5 minutes for 1 hour. To assay process motility, each microglia was analyzed individually across all time points. Time point t=0 minutes was pseudocolored magenta, time point t=5 minutes was pseudocolored green, and the two time points were overlaid to generate the merged image. In this image, purple pixels represent processes which have retracted, green pixels represent pixels which have extended, and white pixels represent processes which remained stable. This analysis was performed for all adjacent pairs of time points. Scale bar = 20μm **B,C**, There is no significant difference in process motility across genotype in either V1 or S1 of p28 mice (n = 8 (V1) or 5 (S1) mice per genotype, one-way ANOVAs; **B**: $p = 0.2235$, $F(2,21) = 1.610$; **C**: $p = 0.3073$, $F(2,12) = 1.304$). **D-G**, There is no significant difference in overall process stability or instability across genotypes in either V1 (**D,F**) or S1 (**E,G**) of p28 mice (n = 8 (V1) or 5 (S1) mice per genotype, one-way ANOVAs; **D**: $p = 0.6816$, $F(2,21) = 0.3904$; **E**: $p = 0.9236$, $F(2,12) = 0.080$; **F**: $p = 0.5780$, $F(2,21) = 0.5627$; **G**: $p = 0.8038$, $F(2,12) = 0.2225$). Graphs show mean ± SEM.

Figure 5.

Loss of CX₃CR1 elicits changes in microglial properties at the ultrastructural level.

A, Representative image of a microglial cell body and processes in V1 of *Cx3cr1*^{G/G} mice at p28. Scale bar = 1 μm. **B**, Inset region of panel **A**. in = inclusion, m = microglia, t = axon

terminal, s = dendritic spine, g = lipofuscin granule. **C-F**, There is no effect of genotype on the number of contacts between microglial processes and dendritic spines, synaptic clefts, or

perisynaptic astrocytic processes, but we found a significant decrease in the number of contacts with axon terminals in *Cx3cr1*^{G/G} mice (n = 3 mice per genotype, Student's t-test;

C: *p* = 0.3559, *t*(4) = 1.043; **D**: *p* = 0.0239, *t*(4) = 3.546; **E**: *p* = 0.2206, *t*(4) = 1.450; **F**: *p* = 0.1832, *t*(4) = 1.608).

G-I, Similarly, there is no effect of genotype on the perimeter of contact between microglia and dendritic spines or axon terminals, but a significant increase

in the perimeter of contact with perisynaptic astrocytic processes is observed (n = 3 mice per

genotype, Student's t-test, **G**: $t(4) = 1.482$, $p = 0.2125$, **H**: $t(4) = 0.9333$, $p = 0.4035$, **I**: $t(4) = 3.777$, $p = 0.0195$). **J**, There is also a significant increase in the number of cellular inclusions within *Cx3cr1*^{G/G} microglial processes (n= 3 mice per genotype, Student's t-test, $t(4) = 9.127$, $p = 0.0008$). Graphs show mean $\pm$ SEM.

Figure 6.

Baseline contact dynamics between microglia and dendritic spines are unaltered in absence of CX₃CR1.

A, Representative images showing contact between microglial processes (yellow) and dendritic spines (green) at 5 minute intervals over 45 minutes. Arrowheads denote putative contacts. Scale bar = 5μm. **B**, There is a significant difference in initial size of spines that are contacted or that remain non-contacted by microglia during the imaging session, with no significant effect of genotype (n = 5 mice per group, two-way ANOVA, main effect of contact $p < 0.0001$, $F(1,24) = 30.44$; genotype $p = 0.1272$, $F(2,24) = 2.250$). **C**, Small spines grow significantly more than large spines after microglia contact spines in all genotypes (n = 5 mice per genotype, two-way ANOVA, main effect of spine size $p < 0.0001$, $F(1,24) = 24.17$; genotype $p = 0.4839$, $F(2,24) = 0.7483$). **D**, There is no significant difference in average contact duration across genotypes (n = 5 mice per genotype, one-way ANOVA, $p = 0.8653$, $F(2,12) = 0.1465$). **E**, There is no significant difference in frequency of contact

across genotypes (n = 5 mice per genotype, one-way ANOVA, $p = 0.8909$, $F(2,12) = 0.1166$). Graphs show mean $\pm$ SEM.

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Figure 7.

Baseline dendritic spine turnover is unaltered in the absence of CX₃CR1.

A, Representative images of dendrites and dendritic spines from *YFP*, *Cx3cr1^{G/+}/YFP*, and *Cx3cr1^{G/G}/YFP* mice. The same segments of dendrites were imaged and analyzed at 3 time points across 4 days (Day 0, Day 2, and Day 4). Arrowheads represent spines which appear or disappear within this period. Scale bar = 5 μm **B**, There is no significant difference in percent of stable, transient, or newly stable spines across genotypes (n = 5 mice per genotype, one-way ANOVAs; stable: $p = 0.2721$, $F(2,12) = 1.454$; transient: $p = 0.5534$, $F(2,12) = 0.6218$; newly stable: $p = 0.4756$, $F(2,12) = 0.7911$). Graphs show mean ± SEM.

Figure 8.

CX₃CR1 is not required for ocular dominance plasticity.

A, Representative images of amplitude maps in V1 generated in response to visual input to each individual eye in p28 mice with intact or ablated fractalkine signaling, before and after 4 days of monocular deprivation. **B**, A significant ocular dominance shift is observed in all genotypes and pharmacological interventions, demonstrating that CX₃CR1 is not required for ocular dominance plasticity (n = 12 mice per genotype, n = 4 mice per antibody group, two-way ANOVA; main effect of deprivation $p < 0.0001$, $F(1,78) = 181.2$, Holm-Sidak post-

hoc * $p < 0.01$, ** $p < 0.0001$; genotype $p = 0.9105$, $F(4,78) = 0.2472$; interaction $p = 0.7766$, $F(4,78) = 0.4438$). Graphs show mean $\pm$ SEM.

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Figure 9.

Microglial behavior during ODP is unaltered in absence of CX₃CR1.

A, Representative images of microglial morphology in binocular primary visual cortex with or without 12 hr monocular deprivation. Scale bar = 50 μm . **B**, Sholl analysis was performed on microglia in C57Bl/6, $Cx3cr1^{G/+}$, and $Cx3cr1^{G/G}$ mice at p28. All genotypes exhibit significant microglial process hyper-ramification after 12 hrs of MD as compared to non-deprived controls (n = 5 animals per genotype, two-way ANOVA; interaction $p < 0.0001$, F

(130, 567) = 8.030; deprivation $p < 0.0001$, $F(5,567) = 197.3$; distance $p < 0.0001$, $F(26,567) = 556.8$. Graphs show mean $\pm$ SEM.

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Figure 10.

Cx3cr1-null mice exhibit a transient defect in developmental microglial infiltration into TCA clusters.

A, Representative images of microglia (green) distribution inside 5-HTT-labelled TCA clusters (red) at p7 and p10 *Cx3cr1^{G/+}* and *Cx3cr1^{G/G}*. Scale bar= 100μm. **B**, *Cx3cr1* null mice have significantly fewer microglial cells inside TCA clusters at p7, but this defect disappears by p10 (n = 6 mice per genotype, two-way ANOVA; genotype $p = 0.002$, $F(1,19) = 12.78$). Graphs show mean $\pm$ SEM.

Figure 11.

CX₃CR1 is not required for eye-specific segregation in the lateral geniculate nucleus.

A, Representative images of contralateral (green) and ipsilateral (magenta) eye retino-geniculate projections (RGPs) in the LGN of C57Bl/6, *Cx3cr1*^{G/+}, and *Cx3cr1*^{G/G} mice at p28. Scale bar= 500μm. **B**, There is no significant difference in the proportion of ipsilateral RGPs in the dLGN across genotypes (n = 6–7 mice per genotype, one-way ANOVA, $p=0.2081$, $F(2,16)=1.734$). **C**, There is no significant difference in the percent of overlap between ipsilateral and contralateral eye RGPs in the dLGN across genotypes across a range of noise thresholds (n = 6–7 mice per genotype, two-way ANOVA, main effect of genotype $p=0.0565$, $F(2,112)=2.948$; interaction $p=0.9955$, $F(12,112)=0.2436$). Graphs show mean $\pm$ SEM.