Undisturbed climbing fiber pruning in the cerebellar cortex of CX3CR1-deficient mice

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Abstract
Pruning, the elimination of excess synapses is a phenomenon of fundamental importance for correct wiring of the central nervous system. The establishment of the cerebellar climbing fiber (CF)-to-Purkinje cell (PC) synapse provides a suitable model to study pruning and pruning-relevant processes during early postnatal development. Until now, the role of microglia in pruning remains under intense investigation. Here, we analyzed migration of microglia into the cerebellar cortex during early postnatal development and their possible contribution to the elimination of CF-to-PC synapses. Microglia enrich in the PC layer at pruning-relevant time points giving rise to the possibility that microglia are actively involved in synaptic pruning. We investigated the contribution of microglial fractalkine (CX3CR1) signaling during postnatal development using genetic ablation of the CX3CR1 receptor and an in-depth histological analysis of the cerebellar cortex. We found an aberrant migration of microglia into the granule and the molecular layer. By electrophysiological analysis, we show that defective fractalkine signaling and the associated migration deficits neither affect the pruning of excess CFs nor the development of functional parallel fiber and inhibitory synapses with PCs. These findings indicate that CX3CR1 signaling is not mandatory for correct cerebellar circuit formation.

Main Points
- Ablation of CX3CR1 results in a transient migration defect in cerebellar microglia.
- CX3CR1 is not required for functional pruning of cerebellar climbing fibers.
- Functional inhibitory and parallel fiber synapse development with Purkinje cells is undisturbed in CX3CR1-deficient mice.

Abbreviations: CB, calbindin; CF, climbing fiber; CTCF, corrected total fluorescence intensity; CX3CR1, CX3C chemokine receptor 1; EGCL, external granule cell layer; EPSC, excitatory postsynaptic current; GCL, granule cell layer; GFP, green fluorescent protein; Igsf9, immunoglobulin superfamily member 9; IHC, immunohistochemistry; KO, knockout; ML, molecular layer; NGS, normal goat serum; P, postnatal day; PBS, phosphate buffer saline; PC, Purkinje cell; PCL, PC layer; PF, parallel fiber; PFA, paraformaldehyde; PPR, paired-pulse ratio; SRC, stimulus–response curve; T, Triton X-100; VGlut2, vesicular glutamate transporter 2; WM, white matter; WT, wild type.

Nicole Kaiser and Christina Pätz contributed equally to this work.
1 | INTRODUCTION

Microglia are the resident phagocytes of the central nervous system and act as the main immune effectors against pathogens. They derive from primitive yolk-sac macrophages and establish during early prenatal development in the brain (Ginhoux et al., 2010; Kierdorf et al., 2013), where they persist throughout the entire life span of the organism by slow turnover (Goldmann et al., 2016).

Resident microglia are constantly scanning their environment for pathological changes (Kreutzberg, 1996; Nimmerjahn, Kirchhoff, & Helmchen, 2005; Schulz et al., 2012). Only upon massive neuropathological challenges, such as irradiation (Mildner et al., 2007) or axonal degeneration (Bechmann et al., 2005), blood-derived mononuclear cells are recruited and differentiate into microglia-like cells (Li et al., 2019; Lund et al., 2018; Shemer et al., 2018).

Besides their function as early sensors for virtually all neuropathological events (Ransohoff & Perry, 2009) additional roles for microglia in the healthy brain during synaptic plasticity and refinement have been described recently (Paolicelli et al., 2011; Schafer et al., 2012; Tremblay, Lowery, & Majewska, 2010). The exact molecular mechanisms regarding microglial-mediated pruning remain unclear. One proven mechanism depends on classical complement cascade elements acting in an activity-dependent manner, which has been described in the retinogeniculate system (Schafer et al., 2012; Stephan, Barres, & Stevens, 2012; Stevens et al., 2007). Complement components C1q and C3 localize to all synapses but microglia preferentially engulf inputs from ‘weak’ synapses for phagocytosis.

Another possible key player of synaptic remodeling, the fractalkine receptor (CX3CR1) signaling, moved into focus of interest. Previous studies revealed that depletion of the fractalkine receptor CX3CR1 results in reduced density of hippocampal microglia accompanied by delayed synaptic pruning (Pagani et al., 2015; Paolicelli et al., 2011). Moreover, microglial physiology may be affected by knockout of CX3CR1 as a deteriorated migration and branch process movement of retinal microglia was observed in mice lacking CX3CR1 (Liang et al., 2009; Paolicelli, Bish, & Tremblay, 2014) and CX3CR1 maturation of glutamate receptor subtypes, such as GluN2A/GluN2B and AMPA/NMDA receptors, is impaired in the somatosensory cortex of knockout mice (Hoshiko, Amoux, Avignone, Yamamoto, & Audinat, 2012).

Synaptic pruning is a developmental program of eliminating surplus synapses (Hua & Smith, 2004; Katz & Shatz, 1996; Sanes & Lichtman, 1999), while other synapses are maintained and strengthened. The process of climbing fiber (CF) maturation in the young postnatal cerebellum is a well-characterized model system and suitable for further investigation into the molecular processes underlying synaptic pruning (Chedotal & Sotelo, 1993; Crepel, Mariani, & Delhaye-Bouchaud, 1976; Kano & Hashimoto, 2009; Mason & Gregory, 1984).

In the immature cerebellum, a given Purkinje cell (PC) is innervated by multiple CFs (Crepel et al., 1976). Within the first three postnatal weeks, CFs undergo a substantial pruning of redundant connections resulting in the typical CF mono-innervation of PCs in the mature cerebellum (Crepel et al., 1976; Mason & Gregory, 1984). After birth, CFs creep among PCs and form several synaptic contacts (Chedotal & Sotelo, 1993) before the dense “nest-type” synaptic connection is established around postnatal Day 5 (Chedotal & Sotelo, 1993; Sugihara, 2005). By P7, CFs enter a stage of functional differentiation, where one of the CFs innervating the given PC is selectively strengthened (Hashimoto and Kano, 2003). The following phase of massive CF elimination proceeds independent (P7–P12) and dependent (P12–P16) on parallel fiber (PF)-to-PC synapse formation resulting in CF-to-PC mono-innervation typically around P14 (Yoshida et al., 2009; Kano & Hashimoto, 2009; Scelfo & Strata, 2005).

In this study, we tested whether the microglial fractalkine receptor CX3CR1, as in the hippocampus (Paolicelli et al., 2011), is a key player for functional pruning of the CF-to-PC synapse. We analyzed whether recruitment of microglia to the site of CF elimination, particularly the PC and molecular layer, as well as the morphological characteristics of microglia are altered in CX3CR1-deficient mice. Further, using electrophysiology, we tested whether CX3CR1-deficient mice show a normal postnatal maturation, that is, the normal elimination of redundant CFs within the first 3 weeks after birth. Additionally, we analyzed whether the formation of PF-to-PC synapses as and GABAergic inhibitory synapses on PCs is normal in mice lacking the CX3CR1 receptor.

2 | MATERIALS AND METHODS

2.1 | Animals

Experiments were performed on mice homozygous for a replacement of the CX3C receptor 1 gene by the enhanced green fluorescent protein (eGFP; CX3CR1−/−, RRID: MGI:2670353, (Jung et al., 2000) and, for controls, on wild-type littermates (CX3CR1+/+) in electrophysiological recordings and C57/BL6N (RRID: IMSR_JAX:000664) mice in immunohistochemical (IHC) analyses and electrophysiological experiments shown. Identification of CFs was done in Igsf9-eGFP transgenic mice (RRID: MMRRC_030804-UCD), a strain in which inferior olive neurons forming CFs express eGFP (Brachendorf, Eilers, & Schmidt, 2015). Animals from postnatal Days (P) 4–21 were used without considering gender. Mice were kept in the animal facility of the University of Leipzig under a 12 hr/12 hr light–dark cycle with access to food and water ad libitum. Animal experimentation was performed in accordance with the EU Directive 2010/63/EU and had been approved by the State Directorate of Saxony, Germany.
2.2 | Genotyping

The genotype of CX3CR1<sup>−/−</sup> and CX3CR1<sup>+/−</sup> mice was confirmed by PCR from tail biopsies using CX3CR forward (TCAGTGTCTCTCCGCTTG) plus CX3CR-WT reverse (CAGTGATCTCTGCGTCCTC) primers or CX3CR forward plus CX3CR-knock in (eGFP) reverse (GTAGTGGTGTGGCGCACGAG) primers, respectively. PCR was performed as follows: one cycle at 95°C of 3 min, 40 cycles comprising 30 s at 95°C, 30 s at 60°C and 1 min at 72°C and one cycle at 72°C of 5 min.

2.3 | Fluorescence immunohistochemistry

Mice were anaesthetized by inhalation of isoflurane (Baxter GmbH, Unterschleißheim, Germany) followed by transcardial perfusion with ice-cold 4% paraformaldehyde (PFA). Cerebella were removed and fixed overnight with 4% PFA. Tissue was subsequently kept in 30% saccharose for 48–72 hr followed by cryo-embedding. The frozen tissue was cut in 50 μm sagittal sections. Blocking was performed with 10% normal goat serum (NGS) and 0.3% Triton X-100 in potassium phosphate buffer saline (PBS) for 1 hr at room temperature (RT). The sections were incubated with primary antibodies for IBA1 (1:500, rabbit, RRID: AB_2661873, Synaptic Systems) and calbindin (1:200, mouse, RRID: 10000347, Swant). All primary antibodies were diluted in PBS containing 0.3% Triton X-100 (PBS/T) and incubated overnight at 4°C. Subsequently, sections were washed twice with PBS/T and incubated with appropriate fluorescent secondary antibodies (Alexa Fluor 568, RRID: AB_2535773 or Alexa Fluor 647, RRID: AB_2633277, 1:400, Life Technologies) in PBS/T for 2 hr at RT kept in the dark. Nuclear counterstaining was performed with DAPI (1:10,000).

Fixed tissue of Igsf9-eGFP mice was cut in 45 μm sagittal sections and immunostained as described previously (Pätz, Brachtendorf, & Eilers, 2018) using primary antibodies for GFP (1:500, goat, biotin conjugated, Rockland, RRID: AB_218204), calbindin (1:1,000, mouse, RRID: AB_1000347), and IBA1 (1:500, rabbit, WAKO, RRID: AB 839504) and the secondary antibodies Streptavidin Cy2 (1:500, Jackson Immuno Research, RRID: AB_2337246), Alexa Fluor 568 (1:200, anti-mouse, RRID: AB 144696), and Alexa Fluor 647 (1:200, anti-rabbit, RRID: AB 1137669), respectively. Unless specified otherwise, all chemicals were obtained from Sigma-Aldrich, Seelze, Germany.

2.4 | Image acquisition and quantification of microglia in the cerebellum

For quantification of microglia, IHC sections were imaged with an inverted confocal laser-scanning microscope equipped with an 40x/1.14 NA oil immersion objective (FW 300, Olympus, Hamburg, Germany) and an upright laser-scanning microscope equipped with an 20x/0.8 NA objective (LSM 800 with Airyscan, Zeiss, Jena, Germany). Tiled images were sampled via motorized stages with a z-step size of 0.5 μm (H117P1, Prior Scientific, Rockland, MA for FW 300 and EK 75 × 50 Pilot, Märzhäuser, Wetzlar, Germany for LSM 800). Images acquired with the FW300 were subsequently combined using Fiji software (Schindelin et al., 2012; Schneider, Rasband, & Eliceiri, 2012) and the stitching plugin (Preibisch, Saalfeld, & Tomancak, 2009). Stitching of the images acquired with the LSM 800 was accomplished with the Zen 2 (blue edition) software (Zeiss). Combining and pseudo-coloring was done in Fiji; linear brightness and contrast adjustments were done homogenously in each combined image.

Cell counting was performed in a standardized region defined as a 200 μm central section within the cerebellar lobulus IV/V including the opposite banks (Figure 1a). Immunostaining of PCs and nuclear counterstaining with DAPI allowed demarcation of the white matter (WM), the granule cell layer (GCL), the PC layer (PCL), the molecular layer (ML) and, in young mice, the external GCL (EGCL, Figure 1b). Microglia were identified by IBA1 staining colocalized with DAPI staining of the nucleus, manually counted and assigned to the respective layers depending on the location of their somata. Assignment to the PCL was done for microglia somata being located one diameter of a typical PC soma above or below the PCL.

Quantitative analysis of microglia morphology (Figures 5 and S1) was done using full volume z-stacks of IBA1-confocal images. Cells without full cell body or processes were excluded. For quantitative analysis of the overall microglial infiltration into the cerebellum (Figures 2 and 3) two to three slices (per animal) were pooled from 5 animals per age and genotype (n = 5). Microglia were then assigned to their position in the respective cerebellar layer. The layer area was measured using the Fiji freehand and polygon selection tool. For visualization of microglial proximity to CF collaterals, one immunostaining of a sagittal cerebellar slice from a P8 Igsf9-eGFP (Fig. 4) mouse (full volume z-stack / 88 slices) is shown.

For the assignment of the morphological index, soma size and arborization area of the microglia (Figures 5 and S1) was quantified by using Fiji freehand and polygon selection tool. The morphological index was calculated as described before (Basilico et al., 2019; Tremblay, Zettel, Ison, Allen, & Majewska, 2012).

2.5 | Electrophysiological recordings

Acute slice preparation from P9 to P17 CX3CR1<sup>−/−</sup>, CX3CR1<sup>+/−</sup>, or C57BL/6 were performed by decapitating and rapidly transferring the brain into ice-cold artificial cerebrospinal fluid (ACSF) saturated with carbogen (95% O₂/5% CO₂). The ACSF included 20 mM Glucose, 125 mM NaCl, 1.25 mM NaH₂PO₄, 26 mM NaHCO₃, 2 mM CaCl₂, and 1 mM MgCl₂ at pH 7.4. Slicing procedure and whole-cell patch-clamp recordings were performed as described previously (Pätz et al., 2018). For extracellular stimulation of CFs, a stimulation electrode was placed in the GCL in 20–100 μm distance to a patched PC. CF activation was assured by an all-or-none response of the first excitatory postsynaptic current (EPSC) to a paired stimulus resulting in a step-wise stimulus-response curve (SRC) and by paired-pulse depression (Konnerth, Llano, & Armstrong, 1990). The number of CFs...
innervating the patched PC was estimated by increasing the stimulation strength, which, in the case of multiple CF innervation, results in a stepwise recruitment of CFs (Bosman, Takechi, Hartmann, Eilers, & Konnerth, 2008). In some recordings, the number of CFs per PC was estimated using two to three independent stimulation electrodes. Here, independent stimulation of individual CFs was ascertained by sequential stimulation of the different CF inputs and absence of paired-pulse depression in crossed stimulations (Bosman et al., 2008; Pätz et al., 2018). To allow proper voltage clamping of CF responses, the normal ACSF solution was supplemented with a submaximal concentration (Pätz et al. 2018; 1 mM) of the rapid glutamate receptor antagonist kynurenic acid. CF and PF responses were acquired in the presence of 10 μM gabazine to block spontaneous GABAergic currents.

For recording of PF paired-pulse behavior (50 ms stimulus interval) and SRGs (repeated every 0.02 s; Figure 6), a stimulation pipette was placed in the ML and PF activation was assured by a graded response of the first EPSC and paired-pulse facilitation (Konnerth et al., 1990). For recording of miniature inhibitory postsynaptic currents (mIPSCs), the pipette solution consisted of (in mM): 114 CsCl, 6 MgCl2, 20 TEA-Cl, 4 ATP-Mg, 10 EGTA, 10 HEPES (osmolality 310 mosm/l) and the ACSF solution was supplemented with 0.3 μM gabazine and 40 μM CNQX.

2.6 | Statistical analysis

Data sets from analyses of IHC images (Figures 2 and 5) were tested for normal distribution using the D’Agostino Pearson normality (Omnibus K2) test (α = .05). Plots show median+interquartile range (25/75). Statistical significances for Figure 2 were determined by Kruskal–Wallis test per age group. Differences between the WT and CX3CR1−/− mice (Figure 3) were determined using multiple unpaired t test with Holm Sidak post hoc correction with α < 0.5. All statistical tests for histological data were conducted using GraphPad Prism 8.1.1 (GraphPad Software, San Diego, CA). Data for microglial characterization (Figure 5) were determined using Wilcoxon rank test with Bonferroni correction per age group.

Data from electrophysiological experiment were tested for normal distribution using the Shapiro–Wilk test (α = .05). Plots show mean+SD in case the majority (>70%) of data were normally distributed (Figure 6), or median+SEM of median otherwise (Figure 8). Differences per age group were tested using the Wilcoxon rank test (Fig. 7) and between WT and CX3CR1−/− mice (Figs. 6 and 8). To test the differences in the graded response of PF-EPSCs between WT and CX3CR1−/− mice (Figure 6b), the slope of the regression lines (in the linear range) of the individual cells was used. All statistical tests for electrophysiological data were done using Igor 8.0 software (Wavemetrics, Lake Oswego, OR). For the analysis of CF-PC synapses (Figure 7), three animals per age group and strain, and two to five PCs per animal were binned (n=3). For the analysis of PF-to-PC synapses 21–22 (Figure 6a) or 15 (Figure 6b) cells from n=5 WT and CX3CR1−/− mice, respectively, were pooled and for of inhibitory synapses 28 cells from n=14 C57/BL6N mice and 25 cells from n=7 CX3CR1−/− mice were binned (Figure 8). Asterisks always denote differences at α levels of ≤0.05 (*), ≤0.01 (**), ≤0.001 (***) and ≤0.0001 (****).

3 | RESULTS

3.1 | Postnatal enrichment of microglia cells in the cerebellar PCL

Earlier studies documented microglial infiltration of the cerebellum during embryonic to early postnatal development describing a layer-
FIGURE 2  Microglial population of the PC and molecular layer peaks within a narrow time window during postnatal development.

(a–c) Example immunostainings of PCs and microglia in sagittal sections of the cerebellar lobule IV/V at postnatal age, P6 (a), P8 (b), and P21 (c). PCs were stained with calbindin (CB, red), microglia with IBA1 (green), and nuclei with DAPI (blue). Note the enrichment of IBA1⁺ microglia in the PCL around P6 and P8. Scale bars 50 μm. (d–i) Density of IBA1⁺ microglia in the cerebellar white, and the gray matter as well as the granule cell, the Purkinje cell, molecular, and the external granule cell layer during postnatal development (P4–P21, wild type). Note that the EGCL was detectable only until P15 and that the ML was detectable from P8 onward. Data are presented as median + interquartile range (IQR) (n = 5). Asterisks denote statistical differences between the ages (Kruskal–Wallis test per age group, *p ≤ .05, **p ≤ .01, ***p ≤ .001, ****p ≤ .0001).
specific heterogeneity in the cerebellar cortex (Ashwell, 1990; Vela, Dalmau, González, & Castellano, 1995) with features of phagocytotic activity such as phagocytotic cup formation (Perez-Pouchoulen, Van-Ryzin, & McCarthy, 2015). We addressed the question whether microglia become specifically enriched nearby CF collaterals, that is, in the PCL, during the period in which excess CF synapses undergo pruning. To this end we quantified the number of microglia cells, stained by IBA1, in P4–P21 wild-type (WT) mice in a standardized area-of-interest in lobulus IV/V (Figure 1).

In the WM, microglia were present at a relatively stable density of around 600 cells per mm² with no significant differences ("n.s.") across postnatal ages (Figure 2d). Microglia density in the gray matter (GM; Figure 2e), on the other hand, showed a clear developmental profile with a peak around P10. Quantification of the layer-specific density revealed a similar developmental profile in the PCL (Figure 2g) and the ML (safely distinguishable from the PCL after P7, Figure 2h; * for an increase in cell number in P8 vs. P12 and P13). The microglia density in the GCL was rather stable (GCL; Figure 2f; n.s.) and the density in the EGCL, which could be resolved up to P15, was negligible (Figure 2i). These data show that microglia accumulate during the pruning period of excess CFs (Hashimoto, Ichikawa, Kitamura, Watanabe, & Kano, 2009) specifically in the areas of the cerebellar cortex in which CFs become eliminated or stabilized, that is, the PCL and ML, respectively.

In order to visualize possible interactions of glia and CFs at a higher resolution we performed IHC analyses in Igsf9-eGFP mice,
3.2 | Delayed population of the granule and ML of CX3CR1 knockout mice during early postnatal development

Earlier studies have shown that lack of the fractalkine receptor CX3CR1 results in reduced microglia density and delayed synaptic pruning in the hippocampus (Pagani et al., 2015; Paolicelli et al., 2011). Moreover, microglial physiology has been suggested to be affected by knockout of CX3CR1 as a deteriorated migration and dynamic motility of processes was observed in retinal microglia lacking CX3CR1 (Liang et al., 2009; Paolicelli et al., 2014). We hypothesized that CX3CR1 signaling may be critically involved in synaptic pruning of CFs and, correspondingly, that deletion of CX3CR1 might impair microglial migration and microglia allocation toward the PCL. Hence, we analyzed the postnatal colonization of the cerebellar cortex in CX3CR1-deficient mice.

While microglia density in the WM of WT mice is rather time-invariant, for CX3CR1−/− mice a slight accumulation of microglia in the WM was observed around P8 (Figure 3b, n.s.). Microglia population of the GM, on the other hand, was delayed during the second postnatal week in CX3CR1−/− mice (Figure 3c, P12). Significantly lower cell numbers were found in the GCL and ML at P12 and P13 (Figure 3d,f), while microglia population of the PCL was fairly comparable in WT and knockout mice (Figure 3e). These data indicate that ablation of CX3CR1 leads to a delayed migration of cerebellar microglia from the white to the GM. Interestingly, the deferred population of the ML was observed at those postnatal ages, at which “winner” CFs begin to stabilize their synapses on dendrites of PCs.

3.3 | Microglial morphology is not affected by deletion of CX3CR1

CX3CR1 has been shown to affect the morphology of microglia in the hippocampus (Pagani et al., 2015). Besides investigating the overall distribution of microglia within the cerebellar cortex, we also analyzed the morphological appearance of microglia during early postnatal development. We calculated the morphological index as the ratio of soma size and arborization area (Basilico et al., 2019) for microglia of WT and CX3CR1−/− mice and binned the cells into three age groups: P5–P8, P9–P13, and P15–P21 (Figure 5).

There was a strong tendency of a declining morphological index during postnatal development of both strains pointing toward the
3.4 | Formation of PF-to-PC synapses is normal in CX3CR1 knockout mice

Previous studies demonstrated that normal formation of PF-to-PC synapses is prerequisite for an undisturbed CF pruning during the late phase of elimination (Kano et al., 1998; Kano & Hashimoto, 2009). Here, we found that around P12, the time at which "winner" CFs begin to stabilize their synapses on PC dendrites, microglia colonization of the ML is delayed in CX3CR1-deficient mice (Figure 4f). To elucidate, whether PF-to-PC synapse formation is affected by this transient reduced number of microglia cells, we tested whether the establishment of functional PF-to-PC synapses is undisturbed in CX3CR1-deficient mice.

To this end, we examined the paired-pulse behavior (50 ms inter-stimulus interval) of PF-EPSCs in P9–P13 CX3CR1+/+ and CX3CR1−/− mice (Figure 5). No significant difference in the paired-pulse ratios was found between WT and KO mice (p = .098, Figure 6a) suggesting that short-term synaptic plasticity and the presynaptic function of PFs seems to be undisturbed in mice lacking CX3CR1. To compare the density of functional PF-to-PC synapses, the SRC of PF-EPSCs was analyzed. Figure 6b displays the graded stimulus intensity-response relation for WT and CX3CR1−/− mice for which data of 15 cells from each five animals per strain were pooled. The amplitudes of LF-MEPSCs increased with rising stimulus intensity similar in WT and KO PCs. No significant difference was found between WT and CX3CR1−/− mice when comparing the slopes of the regression lines of the individual cells (p = .158). Together, these data indicate the functional development of PF–PC synapses is undisturbed in CX3CR1-deficient mice.

3.5 | Pruning of CFs is not affected by CX3CR1 deletion

Previous studies implicated a role for CX3CR1 in the functional maturation and elimination of synapses in the somatosensory cortex and
the hippocampus (Hoshiko et al., 2012; Paolicelli et al., 2011). We, therefore, hypothesized that fractalkine signaling via CX3CR1 may be critically involved in the pruning process of CFs. To this end, we used electrophysiological recordings to analyze CF elimination in WT and in CX3CR1-deficient mice.

We quantified the probability of finding more than one CF per PC in P9–P17 WT and CX3CR1−/− mice. For WT mice, as described previously (Bosman et al., 2008), we found that the switch from multiple- to mono-innervation occurs mostly within 17 days after birth (Figure 7c, left) showing a relatively strong scatter in each age group. A similar pattern was observed in CX3CR1−/− mice (Figure 7c, right), with no significant differences compared to WT mice. Thus, CF elimination proceeds normally in CX3CR1-deficient mice, arguing against CX3CR1 signaling playing a dominant role in CF pruning.

### 3.6 | Inhibitory transmission is not affected by CX3CR1 deletion

Recently, microglia have been shown to be crucial for the development of functional inhibitory synapses on PCs (Nakayama et al., 2018) and that the formation and activation of GABAergic inhibitory synapses during the second postnatal week is critically involved in the refinement of CF-to-PC synapses (Nakayama et al., 2012; Nakayama et al., 2018). To test, whether fractalkine signaling via CX3CR1 is critically involved in the formation of GABAergic inhibitory synapses on PCs, we examined mIPSCs in P9–P13 WT and CX3CR1−/− mice. We found that the amplitude as well as the frequency of mIPSCs in CX3CR1−/− mice do not show significant differences compared to WT mice (p = .24 (Figure 8b), p = .59 (Figure 8c)). These results indicate that inhibitory synaptic transmission is normal in CX3CR1−/− mice and, hence, that CX3CR1 is not crucial for the development of functional GABAergic inhibitory synapses on PCs.

### 4 | DISCUSSION

The contribution of microglia-mediated fractalkine signaling via the CX3CR1 receptor in synaptic pruning appears to be heterogeneous throughout different brain regions (Lowery, Tremblay, Hopkins, & Majewksa, 2017; Paolicelli et al., 2011; Reshef et al., 2017). Here, we report that CX3CR1, while relevant for normal microglial population of the cerebellar cortex, is not required for functional pruning of CF-to-PC synapses. Specifically we found that: (a) knockout of CX3CR1 leads to a slightly delayed microglial population of the granule and the ML, (b) lack of CX3CR1 does not affect microglia morphology, (c) the developmental profile of CF elimination is not altered in CX3CR1-deficient mice, and (d) that the formation of functional PF and GABAergic inhibitory synapses on PCs is undisturbed in the absence of CX3CR1.
In this study, we focused on the impact of CX3CR1 deletion on microglial population of the cerebellum and investigated if microglia do contribute to cerebellar circuit development. Glia cells have been associated with pruning-related incorporation and removal of cell debris, comprising CFs collaterals (Song et al., 2008) and postsynaptic elements of PCs (Morara et al., 2001). Here we show, consistent with previous findings (Nakayama et al., 2018; Perez-Pouchoulen et al., 2015), that after birth, microglia translocate from the WM toward the cerebellar cortex (Figure 2). We further show that population of the PCL and the ML increases during early postnatal development peaking around P8–P13 (Nakayama et al., 2018), which coincides with the period of CF pruning in mouse cerebellum (Hashimoto, Ichikawa, et al., 2009; Scelfo & Strata, 2005). At the same time, microglia specifically extend their processes toward PC somata (Figure 4), raising the possibility that microglia indeed participate in the pruning process of CF-to-PC synapses.

Interestingly, microglia seem to rarely direct engulf CFs during postnatal development in the cerebellum raising the possibility that the majority of CFs might not be pruned by direct engulfment but rather indirectly by synaptic mechanisms, in which microglia may be involved (Nakayama et al., 2018). Besides, hippocampal microglia have been shown to modulate presynaptic structures by selective partial phagocytosis and to be able to induce postsynaptic spine head filopodia by selective engulfment of dendritic spine heads (Weinhard et al., 2018). Both functions support the hypothesis that microglia rather not directly phagocyte material in the cerebellum unlike in other brain regions like the postnatal retinogeniculate system (Schafer et al., 2012).

Our findings suggest, that microglia specifically populate the PCL at the time window of CF elimination giving the possibility of their contribution in the developmental elimination of CFs. Whether microglia directly remove presynaptic and/or postsynaptic structures or whether glia-dependent mechanisms stimulate CF elimination indirectly (Nakayama et al., 2018) requires further investigations.

4.2 | CX3CR1 signaling and synaptic pruning

In the healthy brain, expression of the chemokine receptor CX3CR1 is restricted to microglia and macrophages, while expression of fractalkine, CX3CL1, its only known ligand, is confined to selected neurons indicating their contribution in neuron–microglia interaction (Harrison et al., 1998; Hughes, Botham, Frentzel, Mir, & Perry, 2002; Jung et al., 2000; Nishiyori et al., 1998). Here, CX3CR1−/− mice were used to analyze the possible contribution of fractalkine signaling in CF pruning in the developing cerebellum.

CF elimination starts around P7 resulting in CF-to-PC mono-innervation around P14 (Hashimoto, Ichikawa, et al., 2009; Scelfo & Strata, 2005). Regression of CFs has been described by the probability of finding multiple CFs per PC, which is around 90% at P7 and drastically declines to about 30% during the second postnatal week (Bosman et al., 2008) and to <2% in mature animals (Eccles, Llinás, & Sasaki, 1966). Our data show a prolonged probability of finding
multiple innervation in WT mice and CF-to-PC mono-innervation at P17 (Figure 6), which is somewhat later than previously proposed (Hashimoto, Ichikawa, et al., 2009; Scelfo & Strata, 2005). The latter studies were performed on PCs located in rostral lobules II–VI (Hashimoto, Yoshiida, et al., 2009) or unspecified areas (Bosman et al., 2008). Thus, use of different lobules may affect the probability, although the number of CFs in lobules of the caudal and rostral cerebellum are assumed being equal after P7 (Hashimoto et al., 2001).

Here, we show that CX3CR1<sup>−/−</sup> mice did neither show a disturbed pruning of CFs (Figure 6) nor a disturbed migration of microglia toward the PCL at the time of CF elimination (Figure 3). Thus, in contrast to synaptic refinement in the cortex, hippocampus and olfactory bulb (Hoshiko et al., 2012; Paolilcelli et al., 2011; Reshef et al., 2017), elimination of excess CFs does not rely on fractalkine signaling. In view of the weak expression of fractalkine in the cerebellum compared to the other brain areas (Harrison et al., 1998; Nishiyori et al., 1998), CX3CR1 may be a region-specific mediator of synaptic refinement.

4.3 | CX3CR1 signaling and formation of functional synapses

Microglia have been reported to induce synapse formation in, for example, the hippocampus (Lim et al. 2013) and the somatosensory cortex (Miyamoto et al. 2016). In the mouse barrel cortex, microglial CX3CR1 signaling is critical for long-term remodeling of synapses (Gunner et al., 2019). In the cerebellum, microglia have recently been shown to be crucial for normal formation of functional GABAergic inhibitory synapses on PCs during postnatal development (Nakayama et al., 2018). A disturbed inhibitory synaptic transmission, caused by severe reductions of microglia cells, has been associated with an impairment of CF synapse elimination (Nakayama et al., 2018). Furthermore, the CX3CR1 signaling pathway has been reported to affect functional synapse maturation (Hoshiko et al., 2012; Zhan et al., 2014), plasticity (Maggi et al., 2009; Paolilcelli et al., 2011; Rogers et al., 2011), and activity (Heinisch & Kirby, 2009). Here, we report that the number of microglia cells in the granule and ML of CX3CR1-deficient mice is slightly reduced during the second postnatal week (Figure 3). We show that, at the same time, the formation of functional PF and GABAergic inhibitory synapses on PCs, both prerequisites for proper CF maturation (Kano et al., 1997; Kano & Hashimoto, 2009; Nakayama et al., 2012), is normal in CX3CR1-KO mice (Figures 6 and 8). Thus, absence of CX3CR1 and the accompanied transient reduction of cerebellar microglia neither influences the development of PF-to-PC synapses nor the inhibitory transmission on PCs. In line with our results, an undisturbed formation of PF-to-PC synapses was also reported when microglia where almost absent during postnatal development (Nakayama et al., 2018) suggesting that microglia are not crucial for proper formation of PF synapses. The observed normal establishment of cerebellar GABAergic inhibitory synapses in CX3CR1-deficient mice, in line with previously reported data (Nakayama et al., 2018), implies that fractalkine signaling is not involved in microglia-mediated formation of functional inhibitory synapses in the developing cerebellum (Nakayama et al., 2018). Likewise, there is evidence that the development of GABAergic synapses in the hippocampus is not affected by knockout of CX3CR1 (Bertot, Groc, & Avignone, 2019). Taken together, CX3CR1 seems not to be substantial for normal maturation of GABAergic synapses but may have a heterogeneous role in the functional development of glutamatergic synapses in different brain regions (Bertot et al., 2019; Zhan et al., 2014).

4.4 | CX3CR1 signaling and microglial mobility and morphology

Fractalkine has been shown to induce migration of cortical (Maciejewski-Lenoir, Chen, Meng, & Bacon, 1999) and retinal microglia (Zhang, Xu, Liu, & Zhou, 2012). Earlier studies found impaired microglia dynamics in various brain regions of CX3CR1-deficient mice suggesting transient defects such as slowing of movements of microglial processes and reduced migration and infiltration capacity (Gunner et al., 2019; Liang et al., 2009; Lowery et al., 2017; Paolilcelli et al., 2011). Our data on microglial population of the developing cerebellum of CX3CR1<sup>−/−</sup> mice (Figure 3) show that fractalkine signaling modulates the migratory behavior of microglia also in the cerebellum, indicating that fractalkine signaling is universally required for normal microglia migration across all brain regions. Since the observed effects indicate a transient delayed, but otherwise normal population of the different layers of the cerebellar cortex, CX3CR1 seems not to play a major role in populating the cerebellum cortex.

We found that CX3CR1 deficiency does not affect microglia morphology (Figure 5). These results are similar to findings in the retina (Liang et al., 2009). In hippocampal slices and the somatosensory cortex of CX3CR1<sup>−/−</sup> mice, on the other hand, a reduced mobility of microglia has been reported (Hoshiko et al., 2012; Pagani et al., 2015). However, this discrepancy may be explained by the short postnatal time period in which microglia may be affected. Altogether, this study implies that fractalkine signaling plays a region-specific role in microglia migration and morphology and in synaptic refinement. For pruning of CF-to-PC synapses, CX3CR1 is not an essential player.

5 | CONCLUSION

In summary, we report that CX3CR1 deficiency leads to transient defects in microglial population of the cerebellar cortex, but does not impair pruning of excess CF synapses. This suggests that CX3CR1 exhibits a rather heterogeneous role in synaptic remodeling within specific brain regions. Whether microglia contribute to activity-dependent plasticity of CF-to-PC signaling and how fractalkine signaling is involved in these processes remains subject of further investigations.

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

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