Development

Microglia Are Dispensable for Developmental Dendrite Pruning of Mitral Cells in Mice

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Abstract

During early development, neurons in the brain often form excess synaptic connections. Later, they strengthen some connections while eliminating others to build functional neuronal circuits. In the olfactory bulb, a mitral cell initially extends multiple dendrites to multiple glomeruli but eventually forms a single primary dendrite through the activity-dependent dendrite pruning process. Recent studies have reported that microglia facilitate synapse pruning during the circuit remodeling in some systems. It has remained unclear whether microglia are involved in the activity-dependent dendrite pruning in the developing brains. Here, we examined whether microglia are required for the developmental dendrite pruning of mitral cells in mice. To deplete microglia in the fetal brain, we treated mice with a colony-stimulating factor 1 receptor (CSF1R) inhibitor, PLX5622, from pregnancy. Microglia were reduced by >90% in mice treated with PLX5622. However, dendrite pruning of mitral cells was not significantly affected. Moreover, we found no significant differences in the number, density, and size of excitatory synapses formed in mitral cell dendrites. We also found no evidence for the role of microglia in the activity-dependent dendrite remodeling of layer 4 (L4) neurons in the barrel cortex. In contrast, the density of excitatory synapses (dendritic spines) in granule cells in the olfactory bulb was significantly increased in mice treated with PLX5622 at postnatal day (P) 6, suggesting a role for the regulation of dendritic spines. Our results indicate that microglia do not play a critical role in activity-dependent dendrite pruning at the neurite level during early postnatal development in mice.

Key words: barrel; dendrite pruning; microglia; mitral cells; olfactory system

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Significance Statement

Synapse elimination is essential for activity-dependent circuit remodeling in the developing brains of mammals. Recent studies suggested that microglia play a critical role in the synapse elimination in some systems. This study found that microglia are dispensable for the activity-dependent dendrite pruning in developing mitral cells and layer 4 (L4) neurons in the barrel cortex. Thus, microglia are not critical for activity-dependent dendrite pruning at the neurite level during normal developmental process.

Introduction

During the development of the mammalian nervous system, neurons initially form excessive synaptic connections. During early postnatal development, however, neurons undergo activity-dependent circuit remodeling to form functional neuronal circuits: they strengthen some neurites and synapses while eliminating others (Goodman and Shatz, 1993; Katz and Shatz, 1996). Activity-dependent circuit remodeling is also happening during the learning process in

the adults, but at finer-scales (e.g., remodeling of dendritic spines). Elimination of neurites and synapses is critical to establish precise connectivity; however, molecular and cellular mechanisms of the elimination are poorly understood (Wong and Ghosh, 2002; Luo and O'Leary, 2005; Riccomagno and Kolodkin, 2015).

During early development, circuit remodeling often occurs at the neurite level. Both axons and dendrites are pruned during the remodeling process. In the neuromuscular junctions and climbing fiber, Purkinje cell synapses,

for example, multiple axons connect to a single postsynaptic site, but eventually, all but only one axon is pruned (Lichtman and Colman, 2000; Watanabe and Kano, 2011). Similarly, over 20 retinal ganglion neurons connect their axons to a single neuron in the lateral geniculate nucleus in the early postnatal period; however, eventually, all but one to three strong connections are eliminated within two weeks after eye opening (Chen and Regehr, 2000).

In other systems, dendrites are dynamically pruned. Mitral cells in the olfactory bulb initially extend multiple dendrites to multiple glomeruli; however, they eliminate all but one primary dendrite, which is connected to only one glomerulus (Malun and Brunjes, 1996; Lin et al., 2000; Imai, 2014; Aihara et al., 2021; Fujimoto et al., 2023). This process ensures that each mitral cell receive inputs from a single type of olfactory sensory neurons (OSNs) expressing a specific type of odorant receptor. Layer 4 (L4) neurons in the barrel cortex are also known to establish single barrel-specific connectivity through the activity-dependent remodeling process (Erzurumlu and Gaspar, 2012; Nakazawa and Iwasato, 2021). L4 neurons eventually receive inputs from a single whisker. To form a discrete receptive field, both mitral cells and L4 neurons undergo dendrite pruning. In both neurons, NMDA receptor (NMDAR)-dependent synaptic competition mediates the dendrite pruning process. The failure in the dendrite pruning leads to broader receptive fields.

Recent studies indicate that glia play important roles in the activity-dependent synapse elimination. They exhibit phagocytotic activity and mediate synapse elimination during development (Brown and Neher, 2014; Thion et al., 2018; Faust et al., 2021). During the remodeling of retinal ganglion cell axons, microglia are reported to engulf their synapses in an activity-dependent manner (Stevens et al., 2007; Schafer et al., 2012). It is also known that the number of dendritic spines is increased by the genetic or pharmacological depletion of microglia, suggesting its requirement for the spine elimination (Paolicelli et al., 2011). Therefore, it is an attractive possibility that microglia mediate developmental dendrite pruning via its phagocytotic activity. However, it remains unclear whether microglia play a critical role in dendrite pruning during the developmental process.

In this study, we investigated the possible role of microglia in developmental dendrite pruning in mitral cells in mice. To selectively deplete microglia, we administrated a colony-stimulating factor 1 receptor (CSF1R) inhibitor, PLX5622. However, we did not find any apparent defects in the developmental dendrite pruning of mitral cells in mice treated with PLX5622.

Materials and Methods

Animals

All animal experiments were approved by the Institutional Animal Care and Use Committee of Kyushu University. Pregnant ICR mice were purchased from Japan SLC. Mice were kept under a consistent 12/12 h light/dark cycle (lights on at 8 A.M. and off at 8 P.M.). Both males and females were used for our experiments.

Drug administration

Two types of CSF1R inhibitors, PLX3397 (MedChemExpress, #HY-16749) and PLX5622 (MedChemExpress, #HY-114153), were tested to deplete microglia. We injected the inhibitors into pregnant mice intraperitoneally to deliver them to embryos. After birth, inhibitors were injected intraperitoneally into the neonates. Based on pilot experiments (Extended Data Fig. 1-1*B*), PLX3397 was intraperitoneally injected at a dose of 20 mg/kg body weight twice a day for consecutive days to pregnant mothers (before birth) or pups (after birth). In accordance with previous studies (Rosin et al., 2018), PLX5622 was intraperitoneally injected at a dose of 50 mg/kg body weight once a day for consecutive days to pregnant mothers (before birth) or pups (after birth). In our pilot experiments, PLX3397 treatment reduced microglia at ~70%, which was much lower than PLX5622. We, therefore, only analyzed data with PLX5622 treatment.

Plasmids

pCAG-Flpo (3–10 ng/μl; Addgene, #125576), pCAFNF-tdTomato (1 μg/μl; Addgene, #125575), and pCAFNF-PSDΔ1.2-EGFP (1 μg/μl; Addgene, #125581) used for the *in utero* electroporation have been reported previously (Fujimoto et al., 2023).

In utero electroporation

In utero electroporation was performed to label mitral cells at E12, granule cells at E13, and cortical L4 neurons at E13.5 as described previously (Matsui et al., 2013; Mizuno et al., 2014; Figueres-Oñate and López-Mascaraque, 2016; Fujimoto et al., 2023). Pregnant mice were anesthetized with ketamine (64–80 mg/kg) and xylazine (11–14 mg/kg). A total of 2 μl of plasmid solutions were injected into the lateral ventricle and electric pulses (a single 10-ms poration pulse at 72 V, followed by five 50-ms driving pulses at 32 V with 950-ms intervals) were delivered with forceps-type electrodes (3 mm in diameter, LF650P3, BEX) and a CUY21EX electroporator (BEX).

Preparation of mouse brain samples

Mice were deeply anesthetized by intraperitoneal injection of pentobarbital (Sankyo, #P0776) and perfused with 4% paraformaldehyde (PFA) in PBS. The brain samples
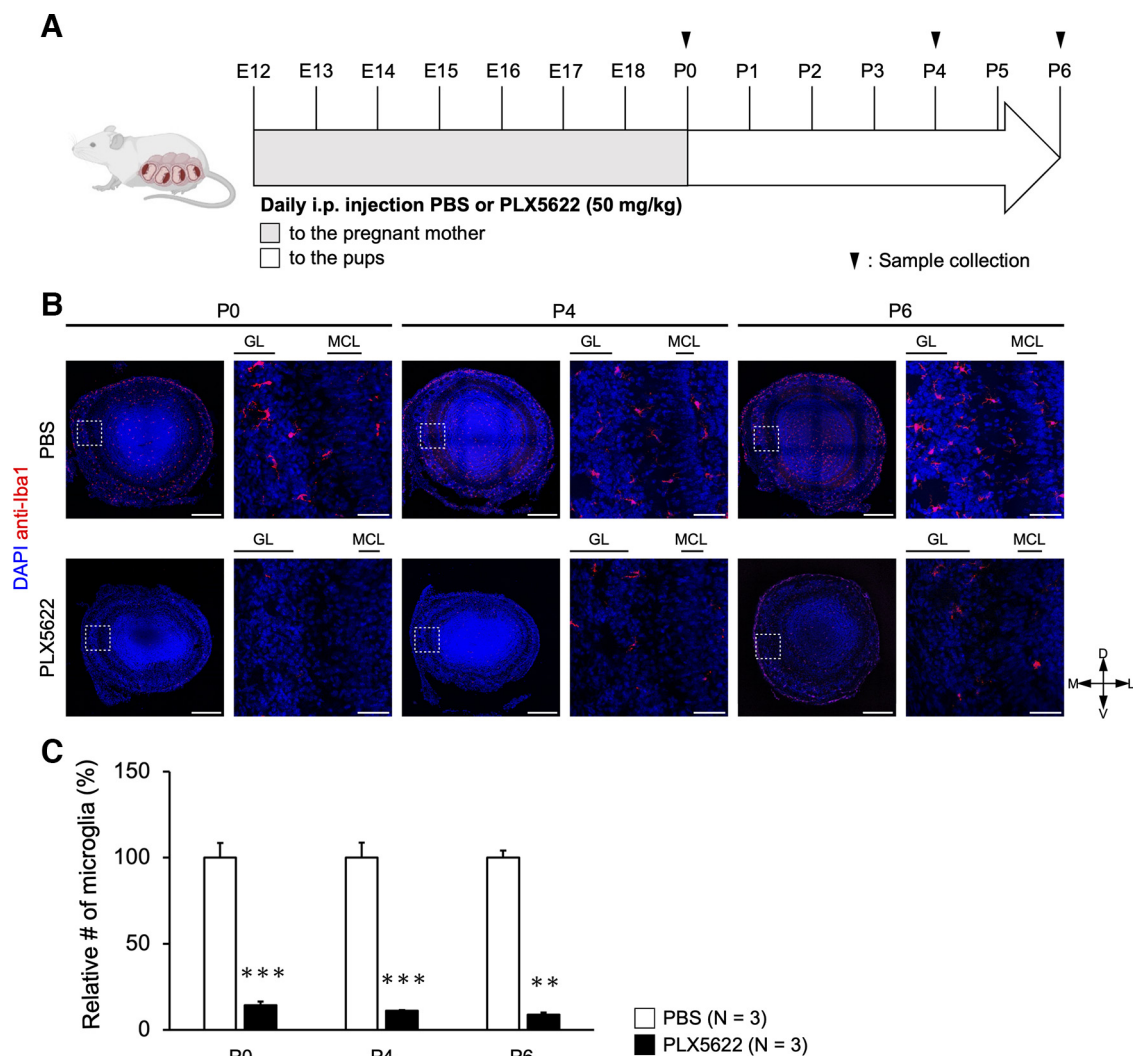
Figure 1. Depletion of microglia with PLX5622. *A*, Timeline of PLX5622 administration. PLX5622 was intraperitoneally injected into pregnant mothers once a day from E12 and to the pups once a day from P0. Animals were analyzed at P0, P4, and P6 based on previous studies (Fujimoto et al., 2023). *B*, Distribution of microglia in the olfactory bulb of control (top) and PLX5622-treated (bottom) mice. Anti-Iba1 staining shows microglia. GL, glomerular layer; MCL, mitral cell layer. Scale bars are 300 and 50 μm. D, dorsal; V, ventral; M, medial; L, lateral. *C*, Quantification of Iba1-positive microglia in the olfactory bulb. One olfactory bulb per animal was analyzed. Every three sections (i.e., every 48 μm) were analyzed for each olfactory bulb for quantification (28–56 sections of 16-μm thickness per animal). Data are from three mice each. ***$p < 0.001$, **$p < 0.01$ (Welch's *t* test). See also Extended Data Figures 1-1 and 1-2 for pilot experiments with PLX3397.

were fixed with 4% PFA/PBS overnight at 4°C. We embedded brain samples in 4% agarose in PBS and prepared brain slices (520 μm thick). To prepare cryosections, we incubated brain samples overnight in 20% sucrose and then in 30% sucrose with a gentle shake and embedded the samples in O.C.T. compound. Coronal frozen sections (16 μm thick) were prepared with a cryostat (Leica #3050S).

Immunohistochemistry

Frozen sections were blocked with 5% skim milk and 1% Triton X-100 in PBS for 1 h at room temperature. Then, the sections were incubated with primary antibodies overnight at 4°C. Rabbit anti-Iba1 (1:1000, Wako, #019-19741), goat anti-OMP (1:500, Wako, #019-22291), rabbit anti-Homer1 (1:200, Synaptic Systems, #160003) were used as primary antibodies. After washing in PBS,

sections were incubated with Alexa 555 or Alexa 488-conjugated donkey anti-rabbit or goat IgG (1:1000 for anti-Iba1 and 1:250 for others; ThermoFisher, #A31572, #A21432, and #A21206) for 2 h at room temperature. Nuclei were stained with DAPI (DOJINDO, #D523). To label a subset of olfactory sensory neurons, biotin-conjugated Lectin from Dolichos biflorus (1:1000, Sigma, #L6533-5MG) was used. Alexa Fluor 488-conjugated streptavidin (ThermoFisher, #S32354) was used to visualize the lectin signals.

For the analysis of the barrel cortex, 120-μm-thick tangential vibratome sections were prepared. The brain slices were incubated in blocking solution (2% saponin, 0.25% fish gelatin, 0.5% skim milk, 0.5% Triton X-100 and 0.05% sodium azide in PBS) overnight. Slices were then incubated with Guinea pig anti-VGluT2 (1:500, Millipore, AB2251-I) diluted in the blocking solution for 2 d at room temperature. After
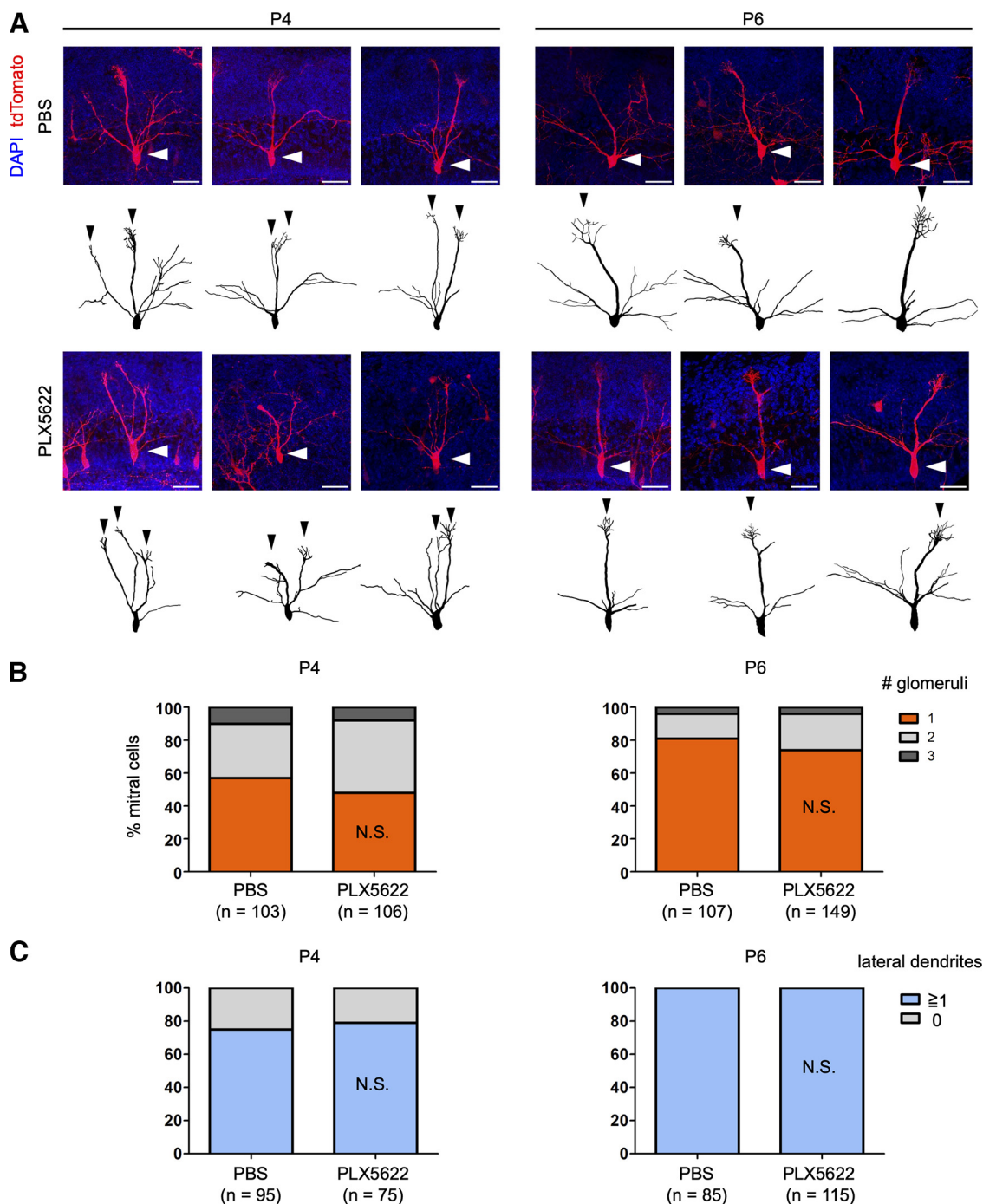
Figure 2. Microglial depletion does not affect dendritic pruning of mitral cells. **A**, Representative images and reconstructions of mitral cells in control and PLX5622-treated mice at P4 and P6. White and black arrowheads indicate somata and primary dendrites of mitral cells, respectively. Scale bars represent 50 μm. **B**, Quantification of the number of glomeruli innervated by single mitral cells. **C**, Quantification of lateral dendrite formation. In this quantification, we excluded mitral cells whose lateral dendrites were densely labeled and difficult to trace. N.S., nonsignificant (χ^2 test compared with the control). Number of neurons (n) are indicated in parentheses. Data are from five or six mice per group.

three times washing in 0.1% Triton X-100/PBS for 1.5 h, the slices were incubated with Alexa Fluor 488-conjugated donkey anti-guinea pig IgG (1:500, ThermoFisher, A-11073) diluted in the blocking solution for 2 d. After twice washing with 0.1% Triton X-100/PBS for 2 h, the slices were cleared with SeeDB2G.

Tissue clearing with SeeDB2G

Fixed brain slices were stained with 0.1% DAPI in PBS overnight at room temperature. The brain samples were then cleared with SeeDB2G as described previously (Ke et al., 2016; Ke and Imai, 2018). Cleared samples were mounted with SeeDB2G on a
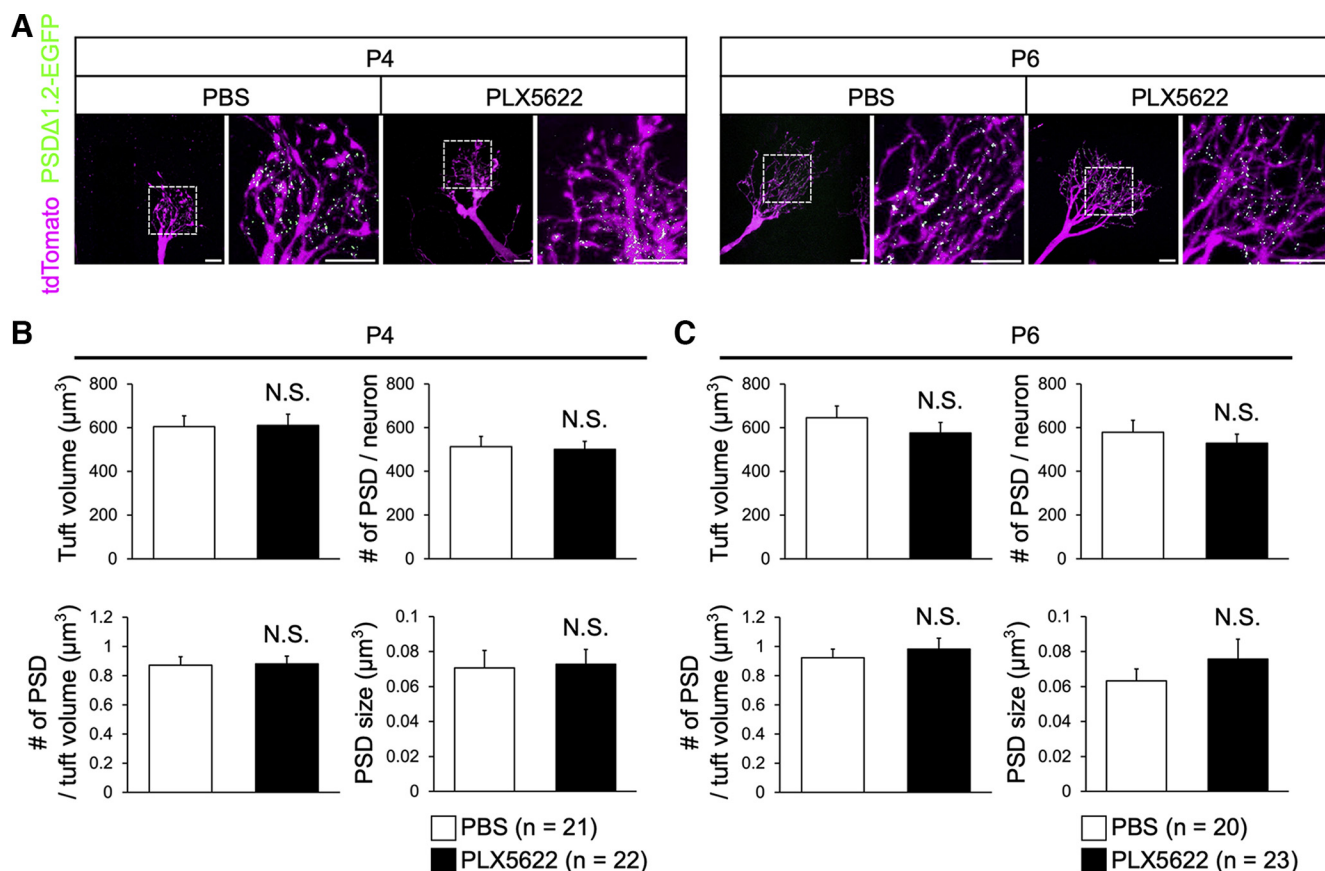
Figure 3. Microglial depletion does not affect the formation of excitatory synapses in mitral cells. **A**, Representative images of dendritic tufts and PSDΔ1.2-EGFP puncta (excitatory synapses) of mitral cells in the control and PLX5622-treated mice at P4 and P6. Images are from glomerular layer. Scale bars represent 10 μm. **B**, **C**, Quantification of dendritic tuft volumes, number of PSD puncta, PSD puncta density, and PSD size at P4 (**B**) and P6 (**C**). N.S., nonsignificant (Welch's *t* test). Number of neurons (*n*) are indicated in parentheses. Data are from five or six mice per group.

glass slide with a 500-μm-thick rubber sheet and a coverslip.

Confocal imaging and image processing

Fluorescence images were taken with a confocal microscope (Leica SP8) with a 20× (HC PL APO 20×/0.75 IMM CORR CS2, NA 0.75) or a 63× (HC PL APO 63×/1.3 Gly CORR CS2, NA 1.3) objective. Pixel size was 0.090 (Figs. 3, 5C), 0.284 (Figs. 1, 6; Extended Data Figs. 1-1C, 1-2B), and 0.568 μm/pixel (Figs. 2, 4, 5A; Extended Data Figs. 1-1E, 1-2D). Z-step size was 0.12 μm (Figs. 3, 5A,C), 1 μm (Figs. 1, 4, 6; Extended Data Figs. 1-1C, 1-2B), and 5 μm (Fig. 2; Extended Data Fig. 1-1E, 1-2D); a 405-nm laser was used for DAPI, a 488-nm laser was used for Alexa488 and GFP, and a 552-nm laser was used for Alexa555 and mRuby3.

Quantification of neuronal morphology and synapses

Mitral cells located at the medial side of the olfactory bulb were analyzed for dendritic morphology following previous studies (Aihara et al., 2021; Fujimoto et al., 2023). Dendrites of mitral cells were reconstructed with Neurolucida (MBF Biosciences). The number of PSDΔ1.2-GFP or Homer1

puncta was counted using an ImageJ plugin, 3D Objects Counter (https://imagej.nih.gov/ij/plugins/track/objects.html).

Statistical analysis

Prism7 (GraphPad) was used for statistical analysis. Data were acquired from at least three animals. Number of neurons (*n*) or animals (*N*) are indicated as a sample size within the figures. Number of animals used in the experiments are indicated in figure legends. Welch's *t* test or χ^2 test was used for statistical analyses.

Data availability

Raw microscopy images have been deposited to SSBD:repository (https://doi.org/10.24631/ssbd.repos.2023.10.326).

Results

Depletion of microglia in the olfactory bulb by PLX5622 treatment

Microglial precursors are known to be generated in the yolk sac and infiltrate into the brain during the embryonic period, where they differentiate and produce microglia (Ginhoux et al., 2010). First, we examined the distribution
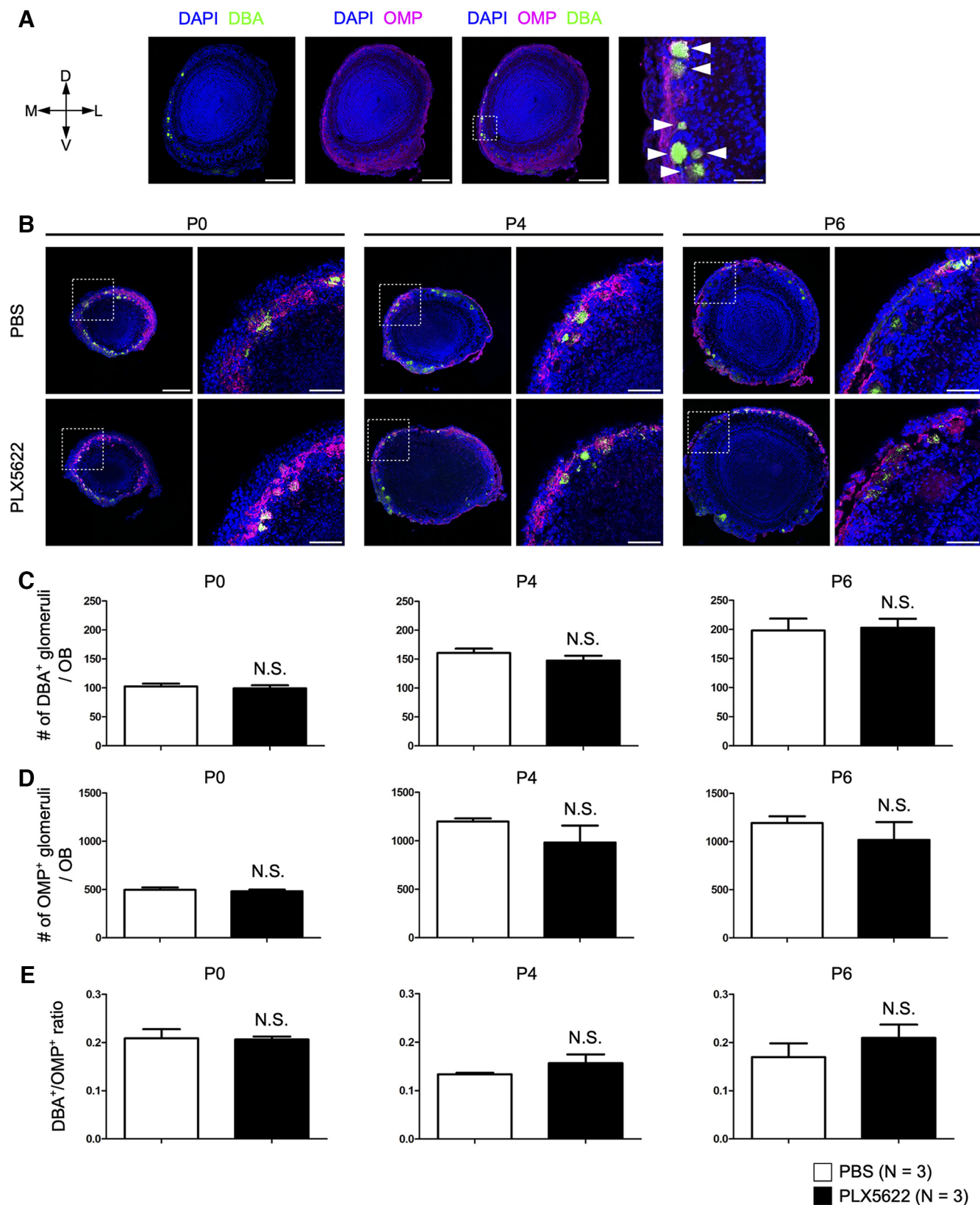
Figure 4. Microglial depletion does not affect axonal projection of OSNs. **A**, Labeling a subset of OSN axons with DBA lectin. Numbers of OMP-positive and DBA-positive (white arrows) glomeruli were quantified. Scale bars are $300\,\mu m$ (left) and $50\,\mu m$ (right). D, dorsal; V, ventral; M, medial; L, lateral. **B**, OSN projection in control (top) and PLX5622-treated (bottom) mice. Scale bars are $300\,\mu m$ (left) and $100\,\mu m$ (right). **C–E**, Numbers of DBA-positive glomeruli (**C**), OMP-positive glomeruli (**D**), and

continued

DBA/OMP ratio (**E**). One olfactory bulb (hemibrain) per animal was analyzed for quantification every three sections (i.e., sampled every 48 μm). N.S., nonsignificant (Welch's *t* test). Number of animals (*N*) are shown in parentheses.

of microglia in the olfactory bulb during the early postnatal stage with anti-Iba1 staining. We found that microglia are widely distributed in the olfactory bulb at postnatal day (P)0, before the dendrite pruning of mitral cells (Fig. 1*B*).

To investigate the role of microglia in the circuit remodeling in the olfactory bulb, we depleted microglia by drug administration. We used PLX5622, a selective and potent inhibitor of colony-stimulating factor 1 receptor (CSF1R), to remove microglia in the brain (Dagher et al., 2015). CSF1R is specifically expressed in microglia in the brain and is essential for microglial survival and proliferation. Therefore, inhibition of CSF1R can specifically deplete microglia (Stanley and Chitu, 2014). PLX5622 administration is known to deplete 80–90% of microglia after 3 d and over 90% after 7 d (Rosin et al., 2018). PLX5622 (50 mg/kg body weight) was intraperitoneally injected into pregnant mother mice from embryonic day (E)12 and intraperitoneally injected into the pups after birth (Fig. 1*A*). PLX5622 was administered once a day for consecutive days. At postnatal days 0, 4, and 6, the number of microglia was analyzed with anti-Iba1 immunostaining. The number of Iba1-positive microglia was reduced by >90% in PLX5622-treated mice at all stages.

We also tested PLX3397, a conventional CSF1R inhibitor (Elmore et al., 2014). We administered PLX3397 during postnatal stages (P0–P6; Extended Data Fig. 1-1) or from early pregnancy (E6–P6; Extended Data Fig. 1-2) at a concentration of 20 mg/kg body weight twice a day for consecutive days. Approximately 70% of microglia were depleted under this condition. Based on these results, PLX5622 was used in subsequent experiments.

Microglial depletion does not prevent dendrite pruning of mitral cells

To examine the dendrite pruning of mitral cells, we employed *in utero* electroporation (Fujimoto et al., 2023). We performed *in utero* electroporation at E12 to label mitral cells. To facilitate neuronal reconstruction, we labeled mitral cells sparsely using a small amount of FLPo plasmid (3–10 ng/μl) and FLP-dependent tdTomato plasmid (1 μg/μl). It has been known that mitral cells initially extend multiple primary dendrites to multiple glomeruli, but between P4 and P6, a majority of mitral cells establish only one primary dendrite connecting to one glomerulus through the dendritic remodeling process (Fujimoto et al., 2023). In this study, olfactory bulb samples at P4 or P6 were analyzed. Samples were cleared with SeeDB2G and imaged with confocal microscopy. The number of glomeruli innervated by individual mitral cells was quantified after Neurolucida reconstruction.

At P4, 57.3% and 48.1% of mitral cells connected primary dendrites to a single glomerulus in control and PLX5622-treated mice, respectively ($p = 0.3466$, χ^2 test). At P6, 81.3% and 73.8% of mitral cells demonstrated single glomerular connection in control and PLX5622-treated mice, respectively ($p = 0.1255$). We did not find statistical

differences at both stages (Fig. 2). It should be noted that a small fraction of mitral cells retains multiple primary dendrites even at later stages (Fujimoto et al., 2023). Consistent results were obtained with PLX3397-treated animals (Extended Data Figs. 1-1, 1-2). It should also be noted that more dramatical pruning defects were found with smaller sample sizes by several genetic manipulations in our previous studies (e.g., NMDAR, RhoA, and BMPR2 knock-outs; Aihara et al., 2021; Fujimoto et al., 2023). Thus, microglia are dispensable for the developmental dendrite pruning in mitral cells.

Microglial depletion does not affect the formation of excitatory synapses in mitral cell dendrites

As we did not find defects in dendrite pruning at the neurite level, we examined possible defects at finer scales. We compared the volume of dendritic tufts within the glomerulus, the number of postsynaptic density (PSD) per neuron, the number of PSD per dendritic tuft volume, and the size of PSD. We used a PSD marker, PSDΔ1.2-EGFP, introduced by *in utero* electroporation (Hayashi-Takagi et al., 2015; Fujimoto et al., 2023). As mitral cells do not have dendritic spines for excitatory synapses, PSDΔ1.2-EGFP is helpful in identifying functional excitatory synapses. However, none of them showed clear defects at both P4 and P6 in PLX5622-treated mice (Fig. 3). Thus, microglia are dispensable for the normal formation and pruning of dendritic tufts and PSDs in mitral cells during the early postnatal stages.

Microglial depletion does not affect the axonal projection of OSNs

In the visual system, microglia are involved in presynaptic pruning. We, therefore, examined whether the projection and refinement of OSN axons are affected by microglial depletion. OSN axons form glutamatergic synapses onto the dendrites of mitral and tufted cells within the glomerulus. We used anti-OMP immunostaining to count the total number of glomeruli. We also examined the convergence and refinement of specific sets of OSNs using Lectin from Dolichos biflorus (DBA-lectin), which labels a subset of OSNs (St John and Key, 2002; Imai et al., 2009). In control animals, only 10–20% of glomeruli are innervated by DBA-positive OSN axons (Fig. 4*A*). We reasoned that the number of DBA-positive glomeruli should increase if the convergence of like axons is severely impaired. However, we did not find differences in the number of total as well as DBA-positive glomeruli by the PLX5622 treatment (Fig. 4*B*). There were no significant differences in the DBA/OMP ratio at all stages ($p = 0.9118$ at P0, 0.2822 at P4, 0.3717 at P6, Welch's *t* test; Fig. 4*C–E*). Thus, the axonal projection of OSNs is largely intact without microglia.

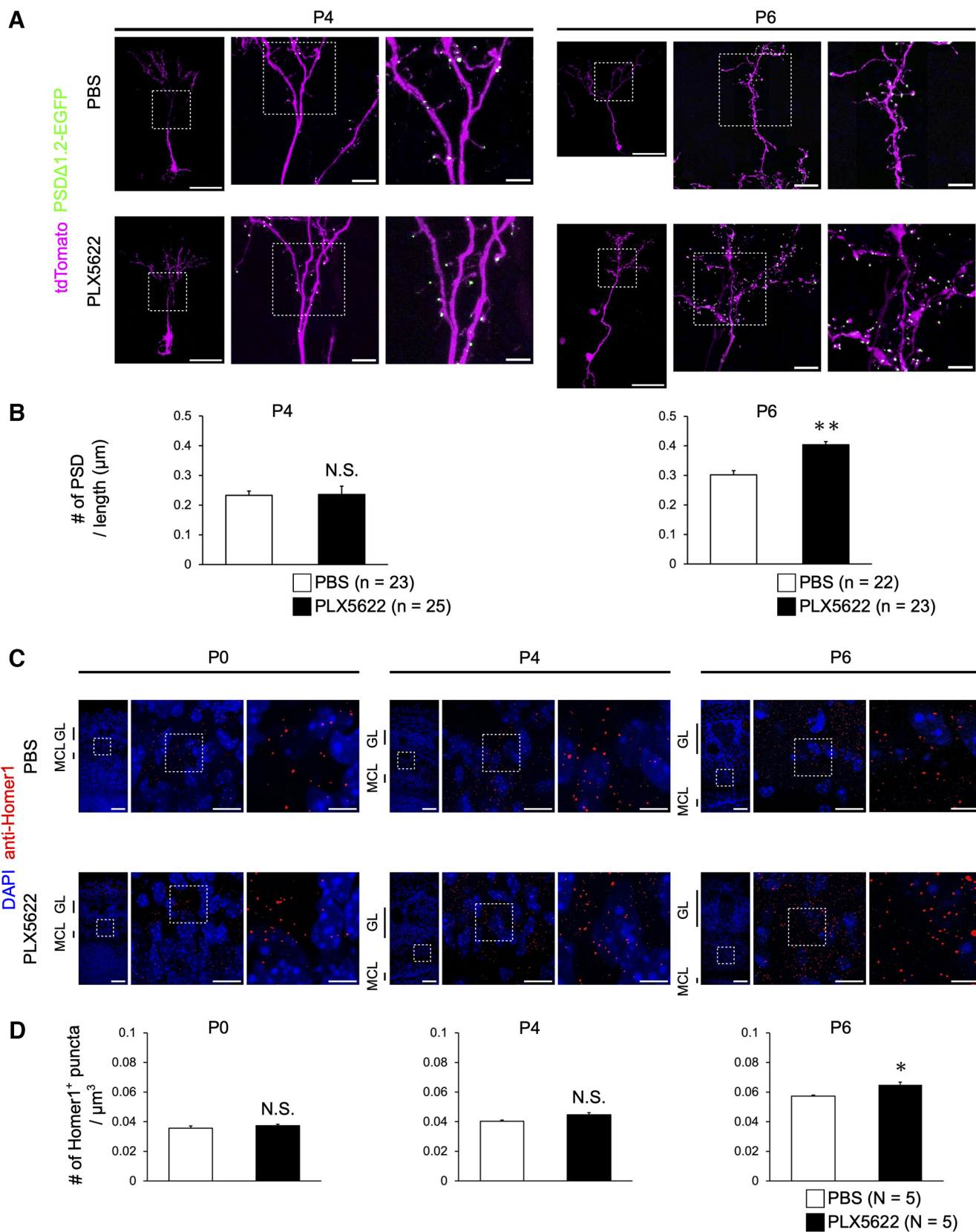**Figure 5.** Microglial depletion increases the number of excitatory synapses in the olfactory bulb granule cells. ***A***, PSDΔ1.2-EGFP puncta in the granule cells in the olfactory bulb of control and PLX5622-treated mice. PSDΔ1.2-EGFP was introduced to a subset of granule cells using *in utero* electroporation at E13. Scale bars represent 50 μm (left), 10 μm (middle), and 5 μm (right). ***B***,

Synaptic development of granule cells is affected by the microglial depletion

In the olfactory bulb, >90% of neurons are inhibitory interneurons, among which granule cells are the most abundant. These interneurons migrate through the rostral migratory stream and are continuously integrated into the olfactory bulb, even in the adult. Lateral dendrites of mitral cells form reciprocal synapses with granule cells in an activity-dependent manner (Kelsch et al., 2009; James et al., 2017). It has been previously reported that microglial depletion in adults leads to a reduction in spine density and elevation of spine head size in adult-born granule cells (Wallace et al., 2020).

We first examined the formation of excitatory synapses at the dendritic spines of granule cells using a PSD marker, PSDΔ1.2-EGFP. We sparsely introduced PSDΔ1.2-EGFP with a filler, tdTomato, using *in utero* electroporation at E13 (Figueres-Oñate and López-Mascaraque, 2016). In this experiment, we labeled a subset of embryonically generated granule cells. PSDΔ1.2-EGFP signals were mostly located at the head of the dendritic spines. At P4, we found no difference in the PSD density between the control and PLX5622-treated mice ($p = 0.9456$, Welch's t test). However, the PSD density was increased by PLX5622 treatment at P6 ($p = 0.0019$, Welch's t test), suggesting that microglia have a suppressive role for excitatory synaptogenesis in granule cells at a later stage (Fig. 5A,B).

To further examine the synaptogenesis in granule cells, we examined the density of Homer1-positive PSD in the external plexiform layer, where granule cell dendrites form excitatory synapses. Again, the Homer1-positive PSD density was significantly increased only at P6 by microglial depletion ($p = 0.7250$ at P0, 0.6878 at P4, and 0.0260 at P6), consistent with the PSDΔ1.2-EGFP labeling experiment (Fig. 5C,D).

Microglial depletion does not affect dendrite pruning in cortical L4 neurons

Lastly, we examined whether microglia are generally dispensable for activity-dependent dendrite pruning. Cortical L4 neurons are a classical model of activity-dependent developmental dendrite pruning (Goodman and Shatz, 1993; Wong and Ghosh, 2002; Matsui et al., 2013). In sensory areas, L4 neurons receive sensory inputs from the thalamus. In mice, L4 neurons in the barrel cortex show clear orientation bias toward the barrel hollow, representing inputs from a specific whisker. Early in the development, dendritic growth is unbiased, but early in the postnatal development, biased orientation is formed through dendrite remodeling. The remodeling process involves the spontaneous activity and NMDA receptor-dependent synaptic competition (Mizuno et al., 2014, 2018; Antón-Bolaños et al., 2019; Fujimoto et al., 2023). Thus, L4 neurons in the barrel cortex are another valuable model to study the dendrite pruning and formation of the discrete receptive field.

We used *in utero* electroporation at E13.5 to sparsely label L4 neurons in the barrel cortex and analyzed their morphology at P10. In both control and PLX5622-treated animals, ~80% of their dendrites were confined within a specific hollow, identified by VGluT2 immunostaining (Fig. 6A–C). Moreover, we found no difference in the total dendritic length and the number of primary branches in L4 neurons (Fig. 6D, E). Thus, microglia are dispensable for the normal dendritic patterning of cortical L4 neurons in the mouse barrel cortex.

Discussion

Roles of microglia in the development of the olfactory bulb circuits

Activity-dependent circuit remodeling is essential for the refinement of the nervous system (Goodman and Shatz, 1993; Katz and Shatz, 1996). It is well understood how neurite outgrowth and stabilization are controlled; however, the mechanisms of the neurite pruning process are still poorly understood. Specifically, the fundamental question is what instructs the pruning process (Lichtman and Colman, 2000; Riccomagno and Kolodkin, 2015). In this study, we examined whether microglia play a critical role in the circuit remodeling in the olfactory bulb.

In the olfactory bulb, developmental dendrite pruning in mitral cells is essential to ensure the one mitral cell – one glomerulus rule, whereby sensory inputs from a single type of odorant receptor are conveyed to each mitral cell in the glomerulus (Imai, 2014). We found that microglial depletion does not perturb the normal dendrite pruning process in mitral cells (Fig. 2). Moreover, we found no change in the formation of excitatory synapses in the dendritic tufts of mitral cells (Fig. 3). Thus, microglia are dispensable for the developmental elimination of neurites and synapses in mitral cells.

In the visual circuits and hippocampus, microglia mediate presynaptic pruning (Faust et al., 2021; Guedes et al., 2022). In this study, we did not find apparent changes in OSN projection by microglial depletion based on DBA and OMP staining (Fig. 4). It is possible, however, that genetic labeling of a specific type of OSNs may reveal finer changes in the axonal convergence of OSNs.

In this study, we observed evident changes by microglial depletion only in the granule cells in the olfactory bulb. At P6, but not P0 or P4, the density of excitatory synapses (dendritic spines) in the granule cells was increased by microglial depletion, suggesting that microglia have a suppressive role in the excitatory synapse development (Fig. 5). As the number of excitatory synapses continued to increase both in the control and PLX5622-treated
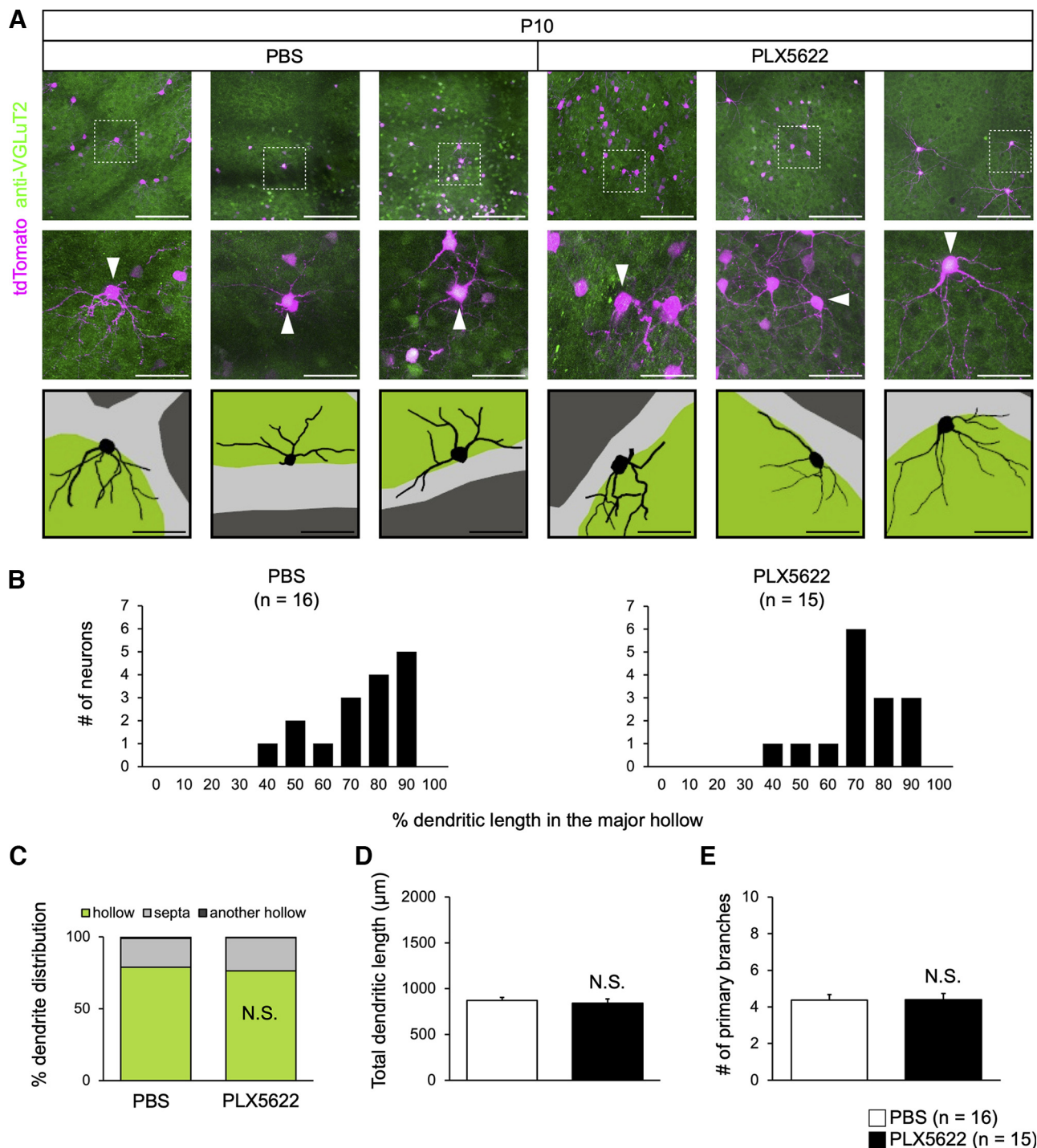
Figure 6. Microglial depletion does not affect the dendrite pruning of L4 neurons in the barrel cortex. **A**, Representative dendritic reconstruction of L4 neurons in the barrel cortex. Barrel hollow (green or dark gray) was identified by anti-VGluT2 immunostaining. White arrowheads indicate somata of analyzed L4 neurons. Scale bars are 150 µm (top) and 50 µm (middle and bottom). **B**, Dendritic orientation. The x-axis shows the percentage of the dendritic length found within the major follow. The y-axis shows the number of neurons. **C**, Quantifications of dendritic orientation. Mean ratios (%) of dendritic length in the major follow (green), septa (light gray), and adjacent hollows (dark gray) are shown. N.S., nonsignificant (Welch's t test). **D**, Quantification of total dendritic length. N.S., nonsignificant (Welch's t test). **E**, Quantification of the number of primary branches. N.S., nonsignificant (Welch's t test). Number of neurons (n) are shown in parentheses. Data are from three mice per group.

mice, it remains unclear whether the increase is because of the reduced synapse elimination or enhanced synaptogenesis. Microglia are reported to facilitate synaptogenesis in some situations (Thion et al., 2018; Guedes et al., 2022); however, we did not find differences in the initial phase of synaptogenesis in granule cells. Our results are consistent with a recent study of adult-born granule cells in the olfactory bulb (Wallace et al., 2020).

It should be noted that astrocytes and oligodendrocyte precursors are also reported to mediate synapse pruning (Chung et al., 2013; Tasdemir-Yilmaz and Freeman, 2014; Auguste et al., 2022; Buchanan et al., 2022, 2023). Therefore, our study does not fully exclude the role of glia and phagocytosis in the dendrite pruning in mitral cells.

We only examined the developmental process in this study. It is possible, however, that microglia play a critical role in circuit remodeling in the adult (Miyamoto et al., 2016), ischemia and injury models (Rappert et al., 2004; Murai et al., 2016), or under neurodegenerative diseases (Guedes et al., 2022) where inflammation by microglia could be more prominent. These are interesting issues for future studies.

It is still debated to which extent glial cells instruct the pruning process (Kettenmann et al., 2011; Riccomagno and Kolodkin, 2015). Glial cells may only eat neurites and synapses which are already committed to the degeneration process.

Mechanisms of developmental dendrite pruning

During development, neurite outgrowth and pruning are both essential to form mature neuronal circuits. It has been suggested that neurite pruning is mediated either by degeneration or retraction (Luo and O'Leary, 2005). Stereotyped large-scale axon pruning is often mediated by degeneration mechanisms (Awasaki and Ito, 2004; Vanderhaeghen and Cheng, 2010; Riccomagno and Kolodkin, 2015). Stereotyped dendrite pruning during *Drosophila* metamorphosis is also mediated by degeneration: before metamorphosis, the stem of the dendrites is thinned by endocytosis, and then the entire dendrite is degenerated by caspase and calpain (Kanamori et al., 2013, 2015). However, this mechanism seems to be unique to insects. Axon pruning in the activity-dependent remodeling process may also involve axon degeneration in mice (Faust et al., 2021; Nagappan-Chettiar et al., 2023). In these cases, the degenerating neurites present the "eat-me" signals, and microglia mediate the clearance of degenerated neurites (Stevens et al., 2007; Schafer et al., 2012; Sapar et al., 2018; Scott-Hewitt et al., 2020; Faust et al., 2021). Microglia is also known to mediate pruning of postsynaptic structures (e.g., dendritic spines; Paolicelli et al., 2011). Indeed, granule cells in the olfactory bulb appear to involve similar mechanisms for synapse elimination (Grier et al., 2016; Denizet et al., 2017; Kurematsu et al., 2022).

In contrast, activity-dependent dendrite pruning at the neurite level in mammals appears to involve a retraction process mediated by actin cytoskeletal regulation. *In vivo* imaging of L4 neurons in the barrel cortex demonstrated that the dendrite patterning results from "trials and errors" of dendritic growth and retraction (Mizuno et al., 2014). Moreover, recent studies showed that Rho-family small GTPases Rac1 and RhoA mediate the stabilization and pruning of mitral cell dendrites, respectively, suggesting that remodeling of the actin cytoskeleton is the key (Aihara et al., 2021; Fujimoto et al., 2023). Indeed, we did not observe the fragmented debris of mitral cell dendrites in the developing olfactory bulb, even in the PLX5622-treated mice (Fig. 2). Our current study indicates that microglia are dispensable for activity-dependent dendrite pruning at the neurite level at this stage. Thus, microglia mediate only a specific type of synaptic pruning.

It is known that the activity-dependent pruning of neurites and synapses is a result of synaptic competition. In synaptic competition, a postsynaptic cell decides which inputs to maintain and eliminates all the others as losers based on the interplay between neurites (Fujimoto et al., 2023). Once the loser postsynapses are determined, the retrograde signals may facilitate the degeneration of input axons (Nagappan-Chettiar et al., 2023). Thus, we assume that intracellular signals within the postsynaptic cell instruct neurite elimination, not only in postsynapses, but also at presynapses. Elucidating the core molecules for synaptic competition should reveal the origin of the pruning specificity in neural development.

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