

Morphological changes in microglia in the mouse brain during postnatal development and obesity

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Summary. Microglia are innate immune cells in the central nervous system (CNS) and play critical roles in proper brain development and function. During postnatal development, microglia have a highly plastic morphology and change rapidly in response to the temporal brain environment. However, their dynamics and phenotypes during this period are still not fully elucidated. Here, we systematically elucidated microglial density and morphological changes during postnatal development as well as in pathological obese conditions. Our results demonstrated a spatiotemporal distribution of microglia in different brain regions associated with gradually increased microglial complexity during postnatal development. Moreover, microglia become reactive in most brain regions of obese mice, but their morphological diversity has a region-specific manner, with an obvious alteration in the hypothalamus. Overall, our data emphasized the morphological dynamics of microglia following developing time windows and provided the basic information for future investigations.

Key words: Microglia, Morphology, Development, Obesity

Introduction

As resident immune cells in the central nervous system (CNS), microglia play multiple functions in developing and adult brains (Borst et al., 2021). They are derived from early erythromyeloid progenitor cells in the

yolk sac around embryonic day 7.25 (E7.25) and subsequently infiltrate into the developing murine CNS at E9.5 until the formation of the blood-brain barrier (Ginhoux et al., 2010). After reaching their final location, microglia start the maturation process by driving broad morphological and transcriptomic changes and become a highly ramified structure (Hammond et al., 2019). Under physiological conditions, microglia can continuously survey the microenvironment of the brain, whereas under pathological conditions, they can rapidly change their morphological characteristics and release inflammatory factors in response to injury (Borst et al., 2021).

In the brain, microglia represent approximately 5-15% of the total cell population (Ransohoff and Cardona, 2010). However, the number and function of microglia are critically controlled by the local environment and neuronal network, leading to a spatiotemporal distribution across distinct brain regions (Orlowski et al., 2003). For example, in the basal ganglia, microglial number in the substantia nigra pars compacta (SNc) is higher than in the ventral tegmental area (VTA). Morphologically, microglia in the nucleus accumbens (NAc) exhibit longer processes and more branches than in the VTA and SNc (De Biase et al., 2017). Additionally, recent evidence showed that the morphology of microglia was inextricably linked to their functions (Kettenmann et al., 2011). In the developing rodent cortex, microglia display amoeboid morphology with large soma and short processes (Vidal-Itriago et al., 2022). This phenotype enables microglia to phagocytose neuronal precursor cells and control the number of neurons in the cortex (Cunningham et al., 2013). Similar to cortical microglia, pineal microglia were also shown to interact with Pax6⁺ precursor cells and modulate the density of Pax6⁺ cells via their phagocytotic activity in the developing pineal gland (Ibanez Rodriguez et al., 2016). Under pathological conditions, microglia could

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undergo morphological and physiological transformations that lead to the release of pro-inflammatory cytokines (Zhan et al., 2014; Cope et al., 2018; Hashemiaghdam and Mroczek, 2020; Wang et al., 2021).

Obesity is a chronic metabolic disorder caused by excessive energy intake, which is associated with increased brain inflammation (Seong et al., 2019; Kawai et al., 2021). Recent studies showed that high-fat diet feeding could induce microglial activation more rapidly, before body weight changes (Thaler et al., 2012; Valdearcos et al., 2019). Interestingly, these reactive microglia were predominantly found in the hypothalamus (Valdearcos et al., 2019), where their role in regulating hypothalamic neural circuitry and synaptic activity has been well described (Cope et al., 2018; Kim et al., 2019). Furthermore, hypothalamic microglia in obese mice have also been reported to release pro-inflammatory factors and subsequently lead to metabolic dysregulation. (Liu et al., 2023).

Understanding the relationship between microglial morphology and their precise physiological function can provide critical insights into the spatiotemporal dynamics of microglial responses. Despite many studies investigating the various roles of microglia, the morphological alterations of microglia across distinct regions of the developing brain are still not fully explored. In the current study, we illustrated a comprehensive morphological analysis of microglia in the mouse brain during postnatal development, as well as in obese mice. Our results indicate a dynamic density of microglia in different brain regions associated with increased morphological complexity during mouse development. In addition, we also found increased microglia number and activity in the genetic *ob/ob* mice compared with wild-type control mice. Together, these data demonstrated that microglia displayed region-dependent morphophysiological diversity in the brains of postnatal and obese mice. The current study provided a useful resource for dissecting microglial contributions to normal development and brain disease.

Materials and methods

Animals

All animal experiments were approved by the Institutional Research Ethics Committee of Xinxiang Medical University (Approved ID: XYLL-20230231) and were executed in compliance with the ethical regulations for animal research. C57BL/6J mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. Mice were selected for the experiment at postnatal day 1 (P1), P7, P14, P28, P42, and P56. Ten-week-old C57BL/6 *ob/ob* mice (B6.Cg-*Lep^{ob}/J*) were purchased from Beijing HFK Bioscience Co., Ltd. Before sacrifice, all animals were housed at 22–25°C in a normal 12h light/dark cycle and given *ad libitum* access to food and water.

Immunohistochemistry and imaging analysis

For immunofluorescent experiments, mice were anesthetized with ketamine/xylazine. Then, mice were transcardially perfused with 20 ml phosphate-buffered saline (PBS) followed by 20 ml 4% paraformaldehyde (PFA). Brains were isolated and post-fixed in 4% PFA overnight. Then, the fixed brain was placed in a DMSO buffer (20% glycerol and 2% dimethyl sulfoxide in 0.125 M PBS) for 24–48h for cryoprotection. Frozen coronal sections (40 µm) were collected in DMSO buffer and kept at -20°C for long-term storage. For immunofluorescence experiments, brain sections were washed three times with 0.125 M PBS, then permeabilized with 0.3% Triton X-100 in 0.125 M PBS for 3h. After blocking, sections were incubated with primary antibodies overnight at 4°C, followed by a two-hour incubation with secondary antibodies. The following antibodies were used: Iba1 (dilution 1:800, Synaptic Systems, 234004, RRID: AB_2493179), CD68 (dilution 1:300, Bio-Rad, MCA1957, RRID: AB_322219), Ki67 (dilution 1:500, Abcam, ab15580, RRID: AB_443209), Goat anti-Guinea Pig IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 488 (1:500 dilution, Thermo Fisher Scientific, A11073, RRID: AB_2534117), Goat anti-Rat IgG (H+L) and Cross-Adsorbed Secondary Antibody, Alexa Fluor™ 568 (1:500 dilution, Thermo Fisher Scientific, A11077, RRID: AB_2534121). Finally, sections were mounted on gelatin-coated glass slides and cover-slipped in Immu-Mount (Thermo Fisher Scientific, 9990414).

Fluorescence images were taken using confocal microscopy (Leica, STELLARIS 5 Cryo, z-stack size 30 µm with z-slice intervals of 1 µm) using 10×, 20×, and 63× objectives at a resolution of 1024×1024 pixels with 8-bit sampling in sequential scanning frame-by-frame mode. One coronal brain section (distance to Bregma: aru-2 mm) per animal was imaged. For quantification of Iba1-positive (Iba1⁺) cells, tile-scanned confocal images using a 10× objective were acquired on the following whole subregions: cortex, hippocampus, thalamus, amygdala, and hypothalamus. The number of Iba1-positive cells in each brain region was obtained by manually counting all Iba1⁺ cells in each region. The regions of interest are depicted in each figure. CD68 intensity per microglia was quantified using Fiji/ImageJ software (National Institutes of Health, version 1.54f), and 6–10 microglia cells were analyzed in each region.

For morphological analysis, images were captured using a Leica STELLARIS 5 Cryo confocal microscope with a 20× objective and a 1.5× zoom factor. Eight–12 microglia per image were analyzed randomly using Fiji/ImageJ software with MotiQ plugins as previously described (Hansen et al., 2022). In brief, single-cell images were first generated by MotiQ cropper and then automatically segmented into binary images by applying an intensity threshold (“Li”-algorithm) using MotiQ thresholder. The skeletonization and quantification of

microglia were performed by the MotiQ 3D analyzer. The cell surface area or cell volume was determined as the total surface area or volume of all remaining 3D particles. The microglial ramification index is defined as the ratio of the cell surface area to the surface area of a perfect sphere with the same volume. Microglial skeleton parameters were generated based on the Fiji/ImageJ plugins “Skeletonize 3D” and “Analyze Skeleton” (Doubé et al., 2010).

Statistical analysis

All data were statistically analyzed using GraphPad Prism 10 software (GraphPad Software). Results are expressed as Mean $\pm$ Standard error of mean (SEM). The experiments were repeated with at least three independent tests. Comparisons between data sets with two groups were evaluated using an unpaired Student's *t*-test. For comparison between three or more groups, one- or two-way ANOVA with Tukey's *post-hoc* test was applied. A *p*-value of less than 0.05 was considered statistically significant.

Results

The morphological dynamics of microglia in the developing cortex

To demonstrate the overall changes in the microglial population in the developing mouse brain, we performed immunofluorescence using an antibody against ionized calcium-binding adaptor molecule 1 (Iba1) on coronal brain sections and counted Iba1⁺ cell numbers across postnatal stages. Results indicated increased Iba1⁺ microglia in the brain, starting from postnatal day 1 (P1), peaking at P14 (Fig. 1A,B). After P28, the number of microglia reduced to around 65% of P14 and remained stable at later stages (Fig. 1A, B).

In the early developmental cortex, microglia present an amoeboid shape and have been reported to modulate neural progenitors through their phagocytic ability (Wright-Jin and Gutmann, 2019). To explore the morphological dynamics of microglia, we then analyzed their number and morphology in the developing cortex. At P1, only a few Iba1⁺ microglia were found in the frontoparietal cortex (Fig. 2A,B). Then, the number of microglia dramatically increased and peaked at P14 (Fig. 2A,B). At P28, the number of microglia was markedly reduced to around 70% of P14 (Fig. 2A,B). There was no difference in microglial number between P42 and P56 (Fig. 2A,B). Morphologically, microglia display a large cell body associated with thick and short processes at P1 (Fig. 2A). As they grow, they gradually adopt a ramified morphology by around P28 (Fig. 2A). ImageJ-based morphological analysis of microglia (Hansen et al., 2022) showed that the ramification index, indicating the complexity of microglia, was gradually increased between P1 and P42 (Fig. 2C). Additionally, the number

of branches, junctions, tips, and tree length, that describe cell skeleton parameters, were significantly increased from P7 to P56 (Fig. 2D-G). Together, those data suggested the morphological dynamics of microglia during cortical development.

To further examine the reason for the increase in microglial number during the early stages, we investigated microglial proliferation using double immunofluorescence with Iba1 and Ki67. As expected, Ki67⁺ microglia were mainly present in the cortex at P1 and P7 (Fig. 2H-J). Ki67⁺ microglia were sharply reduced at P14 and almost undetectable after P28 (Fig. 2H-J). These data suggested a proliferating state of microglia in the neonatal mouse brain.

Morphological changes in microglia in the developing hippocampus

The rodent hippocampus has been well investigated as a powerful circuit model for learning about memory formation and spatial navigation (Bannerman et al., 2014). Interestingly, microglia have been involved in regulating hippocampal neurogenesis and stress resistance (Zhang et al., 2021). To delineate the microglial changes in the developing hippocampus, we further performed immunofluorescence analysis using Iba1 antibody during the postnatal development of mice (Fig. 3). At P1, the average number of microglia reached 55 cells/mm² in the hippocampus, which was higher than in the cortex at this age (Figs. 2B, 3B). The number of Iba1⁺ microglia was significantly elevated at P7 and peaked at P14 (Fig. 3A, B). Then, microglial numbers rapidly reduced to 60% of their peak levels at P28 and remained stable at later stages (Fig. 3A,B). Morphological analysis of microglia indicated a gradually increased ramification index from P1 to P42 (Fig. 3C-G). The number of branches and junctions was significantly increased at P14 compared with P1, and slightly increased at P56 (Fig. 3D,E). The number of tips was gradually increased from P7 to P56 (Fig. 3F). Total tree length was slightly increased induced within P14 compared with P1, but strongly increased by P42 (Fig. 3G). Quantitative analysis of Iba1⁺/Ki67⁺ cells in the hippocampus revealed a higher number of proliferating microglia at P1, which was significantly reduced at P7 and P14, but no longer detected after P28 (Fig. 3 H-J).

Morphological changes in microglia in the developing thalamus

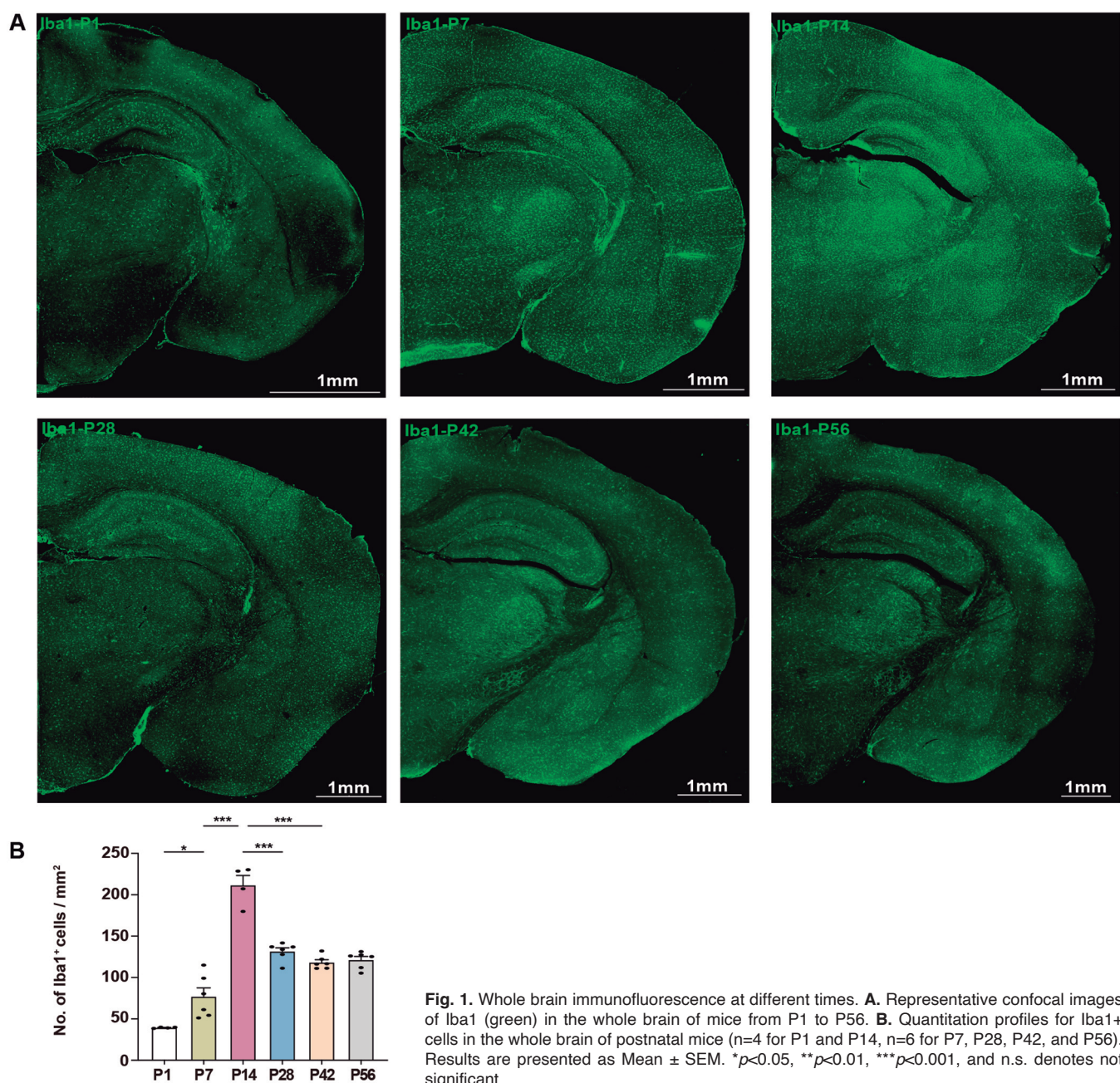
A recent study showed that microglia were involved in the regulation of thalamic neurons and remodeling of emotional neural circuits in mice (Herrero et al., 2002, Liu et al., 2021). To further determine the spatiotemporal dynamics of microglia in the developing thalamus, we also analyzed the microglial phenotype in this area. Compared with P1, the number of microglia was slightly elevated at P7, but sharply increased at P14 (Fig. 4A,B).

After that, the number of microglia gradually decreased until P42 (Fig. 4A,B). In comparison with P1, morphological analyses showed a gradually increased ramification index from P7 to P56 (Fig. 4C). The number of branches, junctions, and tips was stably increased from P7 to P42, and sharply increased at P56 (Fig. 4D-E). In addition, total tree length was increased until P42 and remained stable at P56 (Fig. 4G). Together, these results suggested that microglia in the thalamus have a stably increased microglial complexity following

the developmental stages.

Developing dynamics of microglia in the amygdala

The amygdala is part of the limbic system and is responsible for processing emotion and memory (Janak and Tye, 2015). Microglia have been reported to be involved in the regulation of neuronal activities through synapse phagocytosis in the lateral amygdala and are critical for noise-induced anxiety-like behaviors



Morphological changes in microglia in the mouse brain

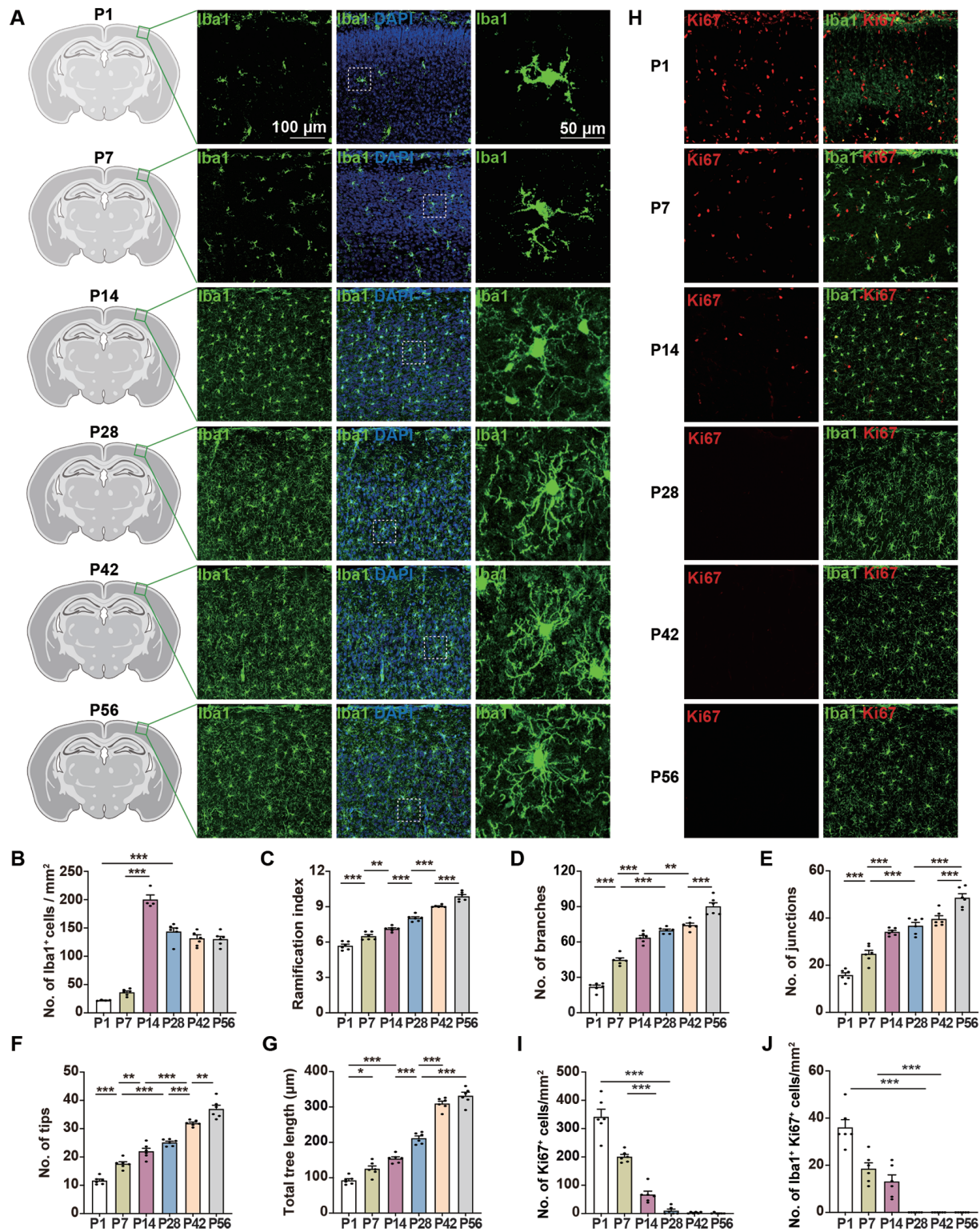


Fig. 2. Morphological changes in microglia in the developing cortex. **A.** Schematic figures of coronal brain sections and representative confocal images of Iba1 immunofluorescence (green) and DAPI (blue) in the cortex of mice from P1 to P56. **B.** Quantitation profiles for Iba1⁺ cells in the cortex of postnatal mice (n=4 for P1 and P14, n=6 for P7, P28, P42, and P56). **C-G.** Morphological analysis of representative cortical microglia from P1 to P56 (n=6): ramification index (**C**), number of branches (**D**), number of junctions (**E**), number of tips (**F**), and total tree length (**G**). **H.** Representative confocal images of Iba1 (green) and Ki67 (red) immunostaining in the developing cortices of mice from P1 to P56. **I, J.** Quantification of Ki67⁺ cells and Iba1⁺/Ki67⁺ cells in the developing cortices from P1 to P56 (n=6). Results are presented as Mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001, and n.s. denotes not significant.

Morphological changes in microglia in the mouse brain

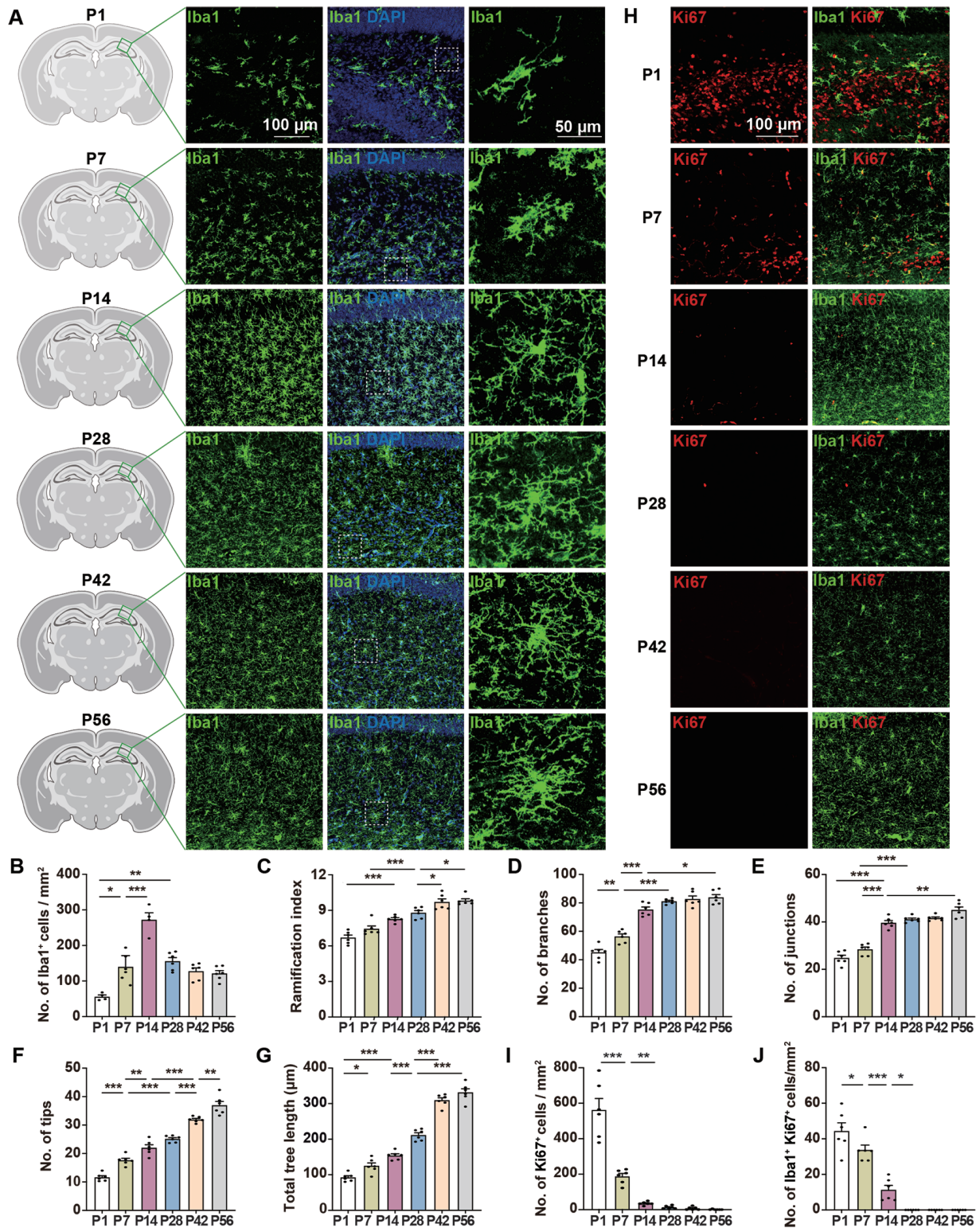


Fig. 3. Morphological changes in microglia in the developing hippocampus. **A.** Schematic figures of coronal brain sections and representative confocal images of Iba1 immunofluorescence (green) and DAPI (blue) in the hippocampus of mice from P1 to P56. **B.** Quantitation profiles for Iba1⁺ microglia cells in the hippocampus of postnatal mice (n=4 for P1 and P14, n=6 for P7, P28, P42, and P56). **C–G.** Morphological analysis of representative hippocampal microglia from P1 to P56 (n=6): ramification index (**C**), number of branches (**D**), number of junctions (**E**), number of tips (**F**), and total tree length (**G**). **H.** Representative confocal images of Iba1 (green) and Ki67 (red) immunostaining in the developing cortices of mice from P1 to P56. **I, J.** Quantification of Ki67⁺ cells and Iba1⁺/Ki67⁺ cells in the developing cortices from P1 to P56 (n=6). Results are presented as Mean $\pm$ SEM. * p <0.05, ** p <0.01, *** p <0.001, and n.s. denotes not significant.

Morphological changes in microglia in the mouse brain

(Acharjee et al., 2018). To explore microglial changes during amygdala development, we further characterized the distribution and morphological features of microglia in the amygdala. Compared with P1, the density of microglia was mildly increased at P7, but strongly increased threefold at P14 (Fig. 5A,B). After reaching maximum cell density at P14, microglial number then significantly reduced and remained stable after P28 (Fig. 5A,B). Through morphological analysis, we found that the arbor complexity of microglia was induced slightly between P1 and P28, but strongly at P42 (Fig. 5C-G).

Similar to the hippocampus, most morphological parameters were gradually increased from P7 onwards and became stable after P42 (Fig. 5D-G). Together, these results suggested that microglia in the amygdala have an increased microglial complexity at later postnatal stages.

Morphological alterations in microglia in the developing hypothalamus

The hypothalamus is located in the ventral brain and functions as a primary region for body homeostasis and

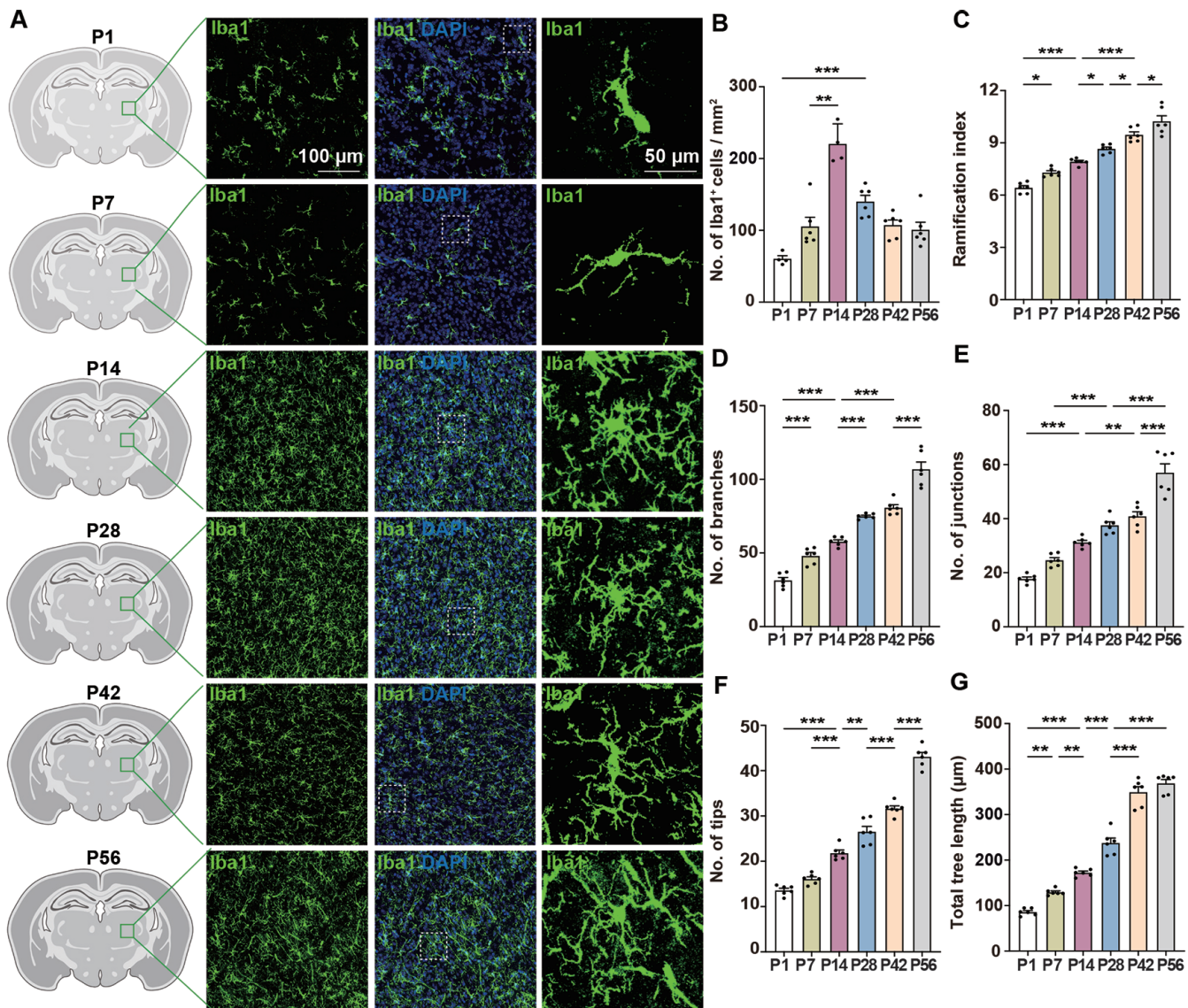


Fig. 4. Morphological alterations in microglia in the developing thalamus. **A.** Schematic figures of coronal brain sections and representative confocal images of Iba1 immunofluorescence (green) and DAPI (blue) in the thalamus of mice from P1 to P56. **B.** Quantitation profiles for Iba1⁺ cells in the thalamus of postnatal mice (n=4 for P1 and P14, n=6 for P7, P28, P42, and P56). **C-G.** Morphological analysis of representative thalamic microglia from P1 to P56 (n=6): ramification index (**C**), number of branches (**D**), number of junctions (**E**), number of tips (**F**), and total tree length (**G**). Results are presented as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s. denotes not significant.

energy metabolism in coordination with the endocrine system (de Lima et al., 2021, Coppola et al., 2022). Microglia have been shown to play a critical role in regulating the plasticity of hypothalamus neurons during postnatal development (Valdearcos et al., 2019, Sun et al., 2024). To validate the dynamic changes in microglia within the hypothalamus, we further analyzed their morphological features in the arcuate nucleus (Arc) of the hypothalamus. Results showed that the number of microglia was similar between P1 and P7, but rapidly increased and peaked at P14 (Fig. 6A,B). Then, results

show microglial number significantly reduced at P28 and remained relatively stable from P28 onwards (Fig. 6B). Double immunofluorescence using Iba1 and Ki67 antibodies indicated a strong microglial proliferation at P1, but quickly reduced after P7 (Fig. 6H-J). No Ki67⁺ microglia were detected after P28 (Fig. 6H-J). By closely tracing the arbor of individual hypothalamic microglia, we found that microglia exhibited an amoeboid morphology with limited processes at P1 (Fig. 6A). All microglial features, including ramification index, branches, junctions, tips, and total tree length, were

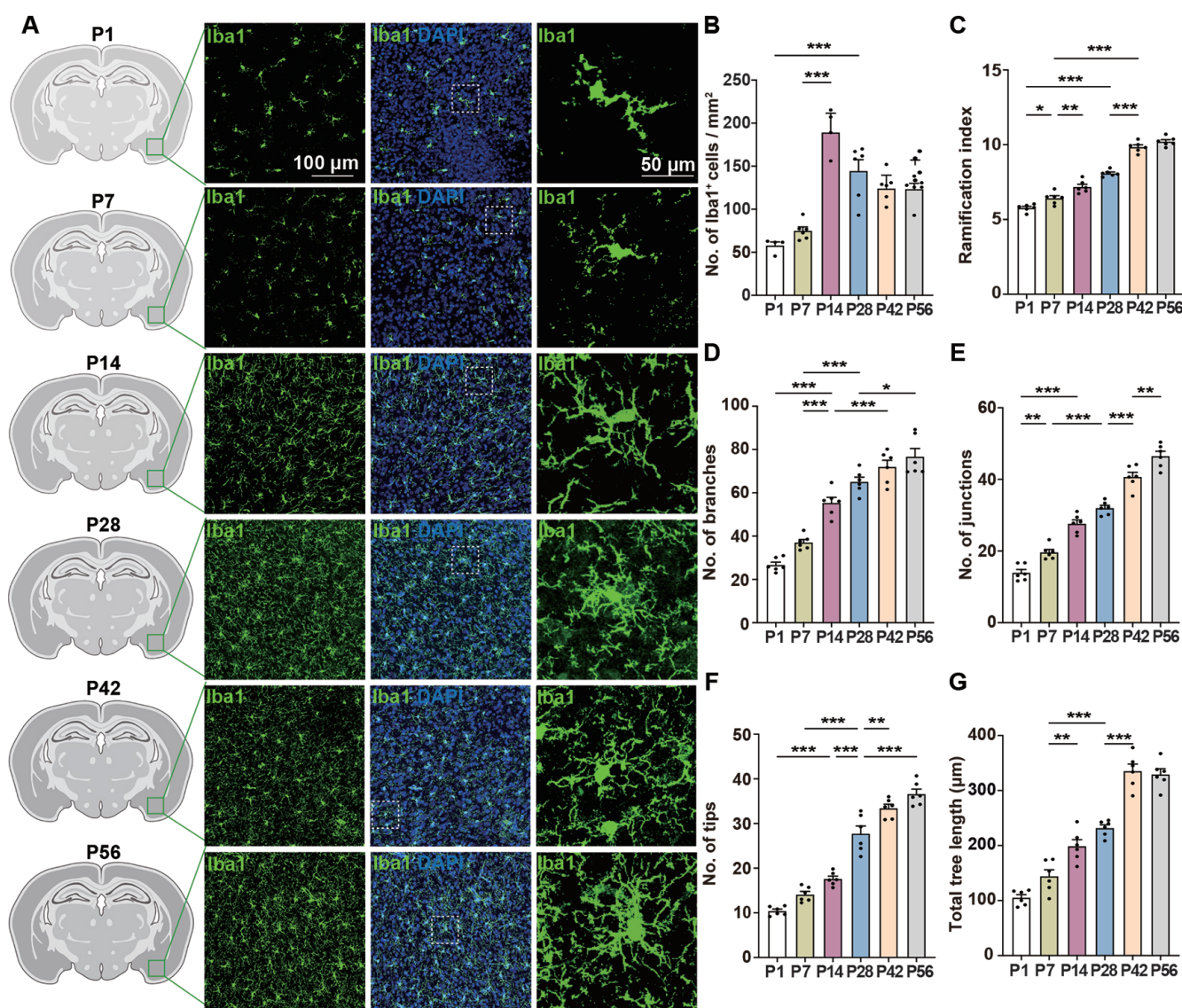


Fig. 5. The morphological dynamics of microglia in the developing amygdala. **A.** Schematic figures of coronal brain sections and representative confocal images of Iba1 immunostaining (green) and DAPI (blue) in the amygdala of mice from P1 to P56. **B.** Quantitation profiles for Iba1⁺ cells in the amygdala of postnatal mice (n=4 for P1 and P14, n=6 for P7, P28, P42, and P56). **C-G.** Morphological analysis of representative amygdala microglia from P1 to P56 (n=6): ramification index (**C**), number of branches (**D**), number of junctions (**E**), number of tips (**F**), and total tree length (**G**). Results are presented as Mean ± SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, and n.s. denotes not significant.

Morphological changes in microglia in the mouse brain

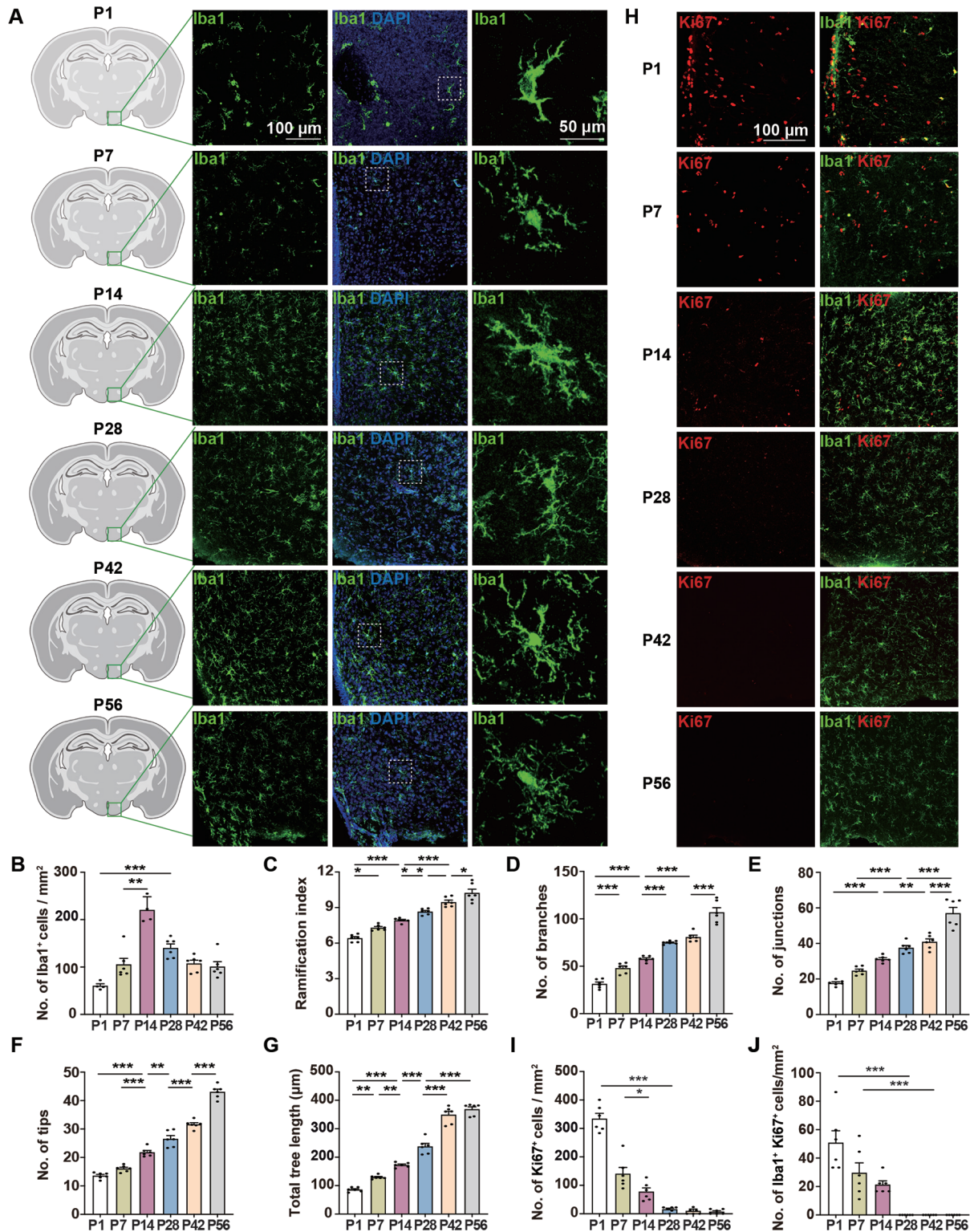


Fig. 6. Morphological alterations in microglia in the developing hypothalamus. **A.** Schematic figures of coronal brain sections and representative confocal images of Iba1 immunostaining (green) and DAPI (blue) in the hypothalamus of mice P1 to P56. **B.** Quantitation profiles for Iba1⁺ cells in the hypothalamus of postnatal mice (n=4, for P1 and P14, n=6, for P7, P28, P42, and P56). **C-G.** Morphological analysis of representative hypothalamic microglia from P1 to P56 (n=6): ramification index (**C**), number of branches (**D**), number of junctions (**E**), number of tips (**F**), and total tree length (**G**). **H.** Representative confocal images of Iba1 (green) and Ki67 (red) immunostaining in the developing cortices of mice from P1 to P56. **I, J.** Quantification of Ki67⁺ cells and Iba1⁺Ki67⁺ cells in the developing cortices from P1 to P56 (n=6). Results are presented as Mean $\pm$ SEM. *p<0.05, **p<0.01, ***p<0.001, and n.s. denotes not significant.

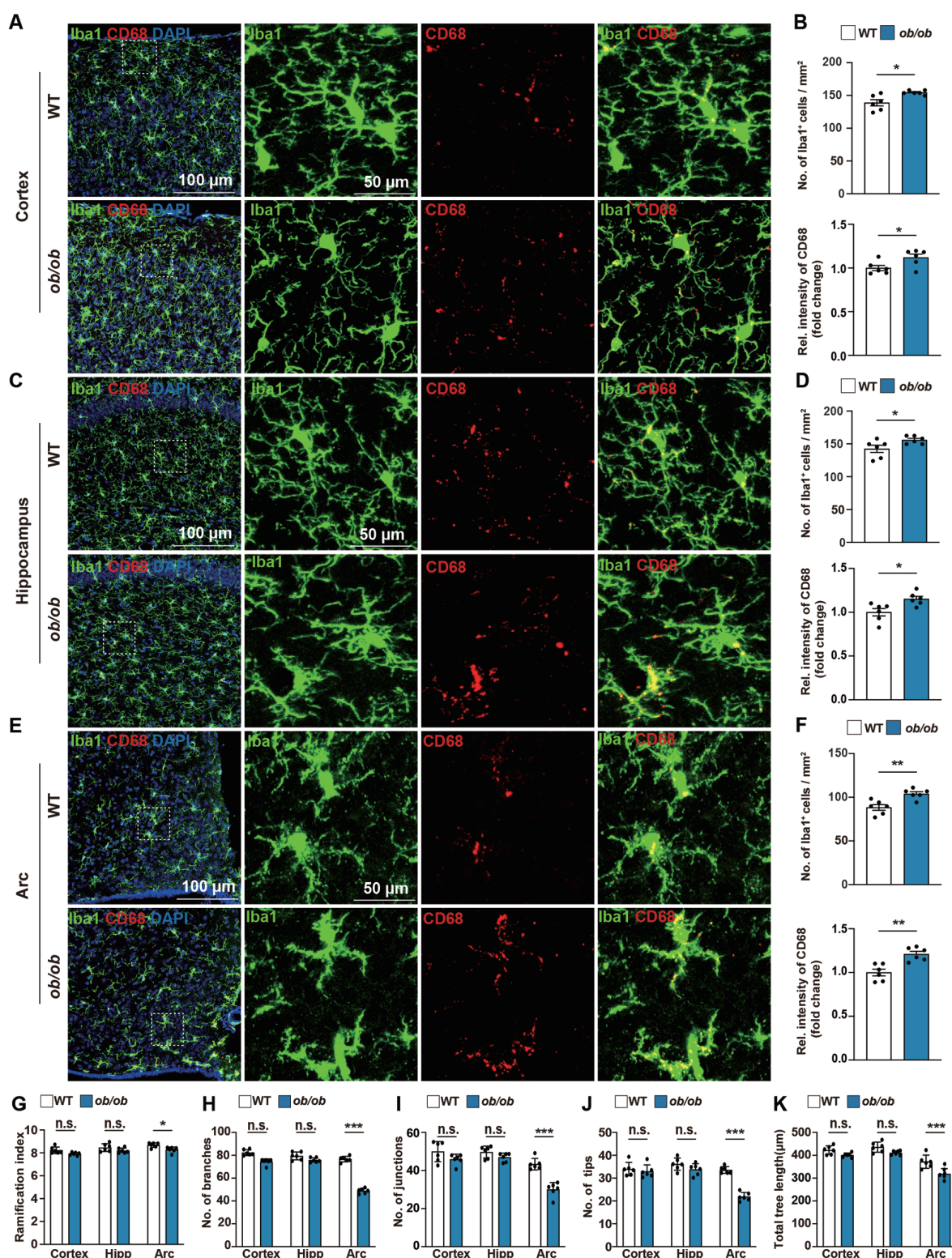


Fig. 7. Reactive microglia in the brain of obese mice. **A, B.** Representative confocal images and quantification of Iba1 (green) and CD68 (red) staining in the cortex of *ob/ob* mice and wild-type controls at 10 weeks of age. **C, D.** Representative confocal images and quantification of Iba1 (green) and CD68 (red) staining in the hippocampus of *ob/ob* mice and wild-type controls at 10 weeks of age. **E, F.** Representative confocal images and quantification of Iba1 (green) and CD68 (red) staining in the hypothalamus of *ob/ob* mice and wild-type controls at 10 weeks of age. **G-K.** Morphological analysis of representative hypothalamic microglia in *ob/ob* mice and wild-type mice: ramification index (**G**), number of branches (**H**), number of junctions (**I**), number of tips (**J**), and total tree length (**K**) (n=6). Results are presented as Mean $\pm$ SEM. * p <0.05, ** p <0.01, *** p <0.001, and n.s. denotes not significant.

steadily increased from P7 and reached maximal levels at P56 (Fig. 6C-G). Together, these data indicated that microglia in the hypothalamus continued their development after birth, with a transition from a prominent amoeboid morphology to a more complex morphology.

Morphological phenotypes in microglia in the brain of obese mice

Obesity is a chronic and systemic inflammation that affects microglial phenotype in the CNS (Maldonado-Ruiz et al., 2017). Recent evidence showed that diet-induced obesity could cause microglial reactivation through NF- κ B signaling, leading to an increased food intake in mice (Valdearcos et al., 2017). To further explore the systemic alterations of microglia in obese mice, we employed *ob/ob* mice, which have a *leptin* gene mutation, and analyzed their microglial phenotypes in different brain regions. By quantification, the density of microglia in *ob/ob* mice was found to be slightly increased in the cortex and hippocampus, but largely increased in the hypothalamus, when compared with the wild-type control (Fig. 7A-B). CD68 is a transmembrane glycoprotein expressed by tissue macrophages (Fiala et al., 2002). In the brain, CD68 is expressed in resting microglia, and its expression increases with microglial activation (Hopperton et al., 2018). Interestingly, the intensity of CD68 was significantly upregulated in microglia of *ob/ob* mice compared with control mice (Fig. 7A-F). To examine morphological changes in microglia, we then analyzed microglia from selected brain regions, including the cortex, hippocampus, and Arc of the hypothalamus. There were no morphological changes in microglia in the cortex and hippocampus (Fig. 7G-K). However, an obvious morphological change was observed in the Arc of *ob/ob* mice (Fig. 7E-K). All morphological features, such as branches, junctions, tips, and total tree length, were found to be strongly reduced in *ob/ob* mice compared with the wild-type control (Fig. 7G-K). Taken together, those results indicated that the morphological changes observed in hypothalamic microglia of obese mice may represent a specific response to the metabolic stress induced by obesity (Wang et al., 2021).

Discussion

During postnatal development, the brain undergoes significant biological and functional maturation, including structural remodeling (Semple et al., 2013). Microglia exhibit dynamic morphological and physiological changes throughout development and actively influence neurogenesis, synaptic pruning, and neuronal apoptosis (Wakselman et al., 2008; Paolicelli et al., 2011; Cserep et al., 2022). In the current study, we extended our understanding of the morphological dynamics of microglia during postnatal development and

demonstrated the spatiotemporal coordination between the growth of the microglial population and their morphological changes. Furthermore, we also analyzed the effects of obesity on the microglial phenotype and indicated region-dependent morphological changes in the obese brain.

The immune cells in the CNS include parenchymal microglia, as well as CNS-associated macrophages, such as meningeal and perivascular macrophages (Prinz et al., 2017). Both microglia and these macrophages are derived from early erythromyeloid progenitors (Mass et al., 2016). Iba1 is a cytoplasmic protein and is primarily restricted to microglia in the brain. In contrast to other homeostatic microglial markers, such as TMEM119 and P2RY12, Iba1 can stably stain ramified and activated microglia and is optimal to display microglial morphologies (Mondo et al., 2020). It was also expressed in meningeal and peripheral macrophages, and hematopoietic cells (Buckman et al., 2014). Under homeostatic conditions, microglia can be distinguished due to their location and morphological differences (Cuadros et al., 2022). However, under pathological conditions, peripheral macrophages can invade the CNS in response to inflammatory stimuli and display microglial features (Li and Barres, 2018). For example, in high-fat diet-induced obesity, peripheral hematopoietic cells could enter the CNS and present as Iba1⁺ cells (Buckman et al., 2014).

In line with previous reports (Alliot et al., 1999; Nikodemova et al., 2015), our data revealed a dramatically increasing microglial density within the first two postnatal weeks, which rapidly decreased in the third week. Microglial density remained relatively stable at later postnatal stages. The reason for the reduced microglial number after P14 was probably due to increased microglial apoptosis during this period (Alliot et al., 1999). Parallely, the density of microglia was lower in the cortex at birth, but sharply increased within the first two postnatal weeks. Notably, recent studies have indicated that the increase in microglia was proportional to the increasing size of the brain (Thompson et al., 2024). In mice, the total brain volume undergoes a rapid expansion after birth and remains stable after three postnatal weeks (Hammelrath et al., 2016). Individually, the volume of the cortex or hippocampus was approximately 40% of their adult volumes after birth and quickly increased to 80% at P14 (Thompson et al., 2024). In contrast, the volume of the hypothalamus at birth had already reached 60% of its adult volume and was almost the same as that of the adult at P14 (Thompson et al., 2024). Therefore, the data suggested that the regional difference in increased microglial number was closely associated with size changes in individual brain regions during early development.

As the principal immune cells in the CNS, the morphology of microglia is highly dynamic and closely related to changes in the surrounding environment (Borst

et al., 2021). During the embryonic stage, microglia often present a round ameboid morphology with a large soma and few thick processes in the rodent neocortex (Cunningham et al., 2013). This phenotype enables microglia to phagocytose mitotic neural precursors and limit the number of neurons in the developing cortex (Cunningham et al., 2013). During postnatal development, microglia have been shown to eliminate excessive synapses between the second and the third postnatal weeks (Lewis, 2011) and regulate the dynamics of synaptic connections in the developing brain (Paolicelli et al., 2011). Additionally, microglia were also reported to participate in removing apoptotic neurons and promoting neuronal survival during development (Vukovic et al., 2012; Ueno et al., 2013). This time-dependent alteration reflects an adaptive transition of microglia from proliferation to maturation in response to environmental changes (Cayre et al., 2009). Together, these data suggest that the morphological dynamics of microglia are critically regulated and essential for brain development.

Obesity is a metabolic disorder and is associated with chronic inflammation (Milanova et al., 2019). Recent evidence indicated that diet-induced obesity increased the microglial population and triggered microglia activation in the hypothalamus, which was associated with morphological changes in microglia (Thaler et al., 2012). Interestingly, these diet-responsive microglia appear to be mainly distributed in the arcuate nucleus instead, but not in other hypothalamic regions (Valdearcos et al., 2014, 2017), indicating a direct effect of saturated fat on microglial activity (Thaler et al., 2012; Cutugno et al., 2024). The activated microglia in the obese hypothalamus could release excessive amounts of pro-inflammatory cytokines and increase toxicity to nearby neurons (Yi et al., 2017), which finally causes loss of Proopiomelanocortin (POMC) neurons and dysfunction of the neuronal network involved in maintaining energy balance (Coupe et al., 2012; Thaler et al., 2012). *ob/ob* mice have a genetic mutation in the *Leptin* gene and exhibit both obese and systemic inflammatory phenotypes (Faggioni et al., 1997; Kosacka et al., 2019). Compared with Diet-Induced Obesity (DIO), *ob/ob* mice present an overall increased microglial number in the cortex, hippocampus, and hypothalamus, associated with elevated CD68 intensity. However, by quantification, morphological changes in microglia are only present in the hypothalamus of *ob/ob* mice, not in other brain regions. These results could reflect a region-dependent response of microglia in *ob/ob* mice. The reason for the morphological differences in microglia within the brain of obese mice is still unclear and requires further investigation.

In conclusion, our analysis provided a basic but comprehensive analysis of morphological changes in microglia during postnatal development and under obese conditions. These findings will be useful for the mechanistic investigation of neurodevelopment and neurological diseases.

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Availability of data and material. All primary data supporting the findings of this study are available upon reasonable request.

Author's contributions. Y.L., C.Z., G.G., and J.K. performed animal experiments, microscopy images, and statistical analysis. Y.L. and C.Z. performed morphometric analysis. Y.L., C.Z., X.Y., L.Q., and J.L. wrote the manuscript. L.Q., X.Y., and J.L. contributed to the conception and design of the study. All authors approved the final version of this manuscript.

Conflict of Interest. The authors declare no competing interests.

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