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Microglial numbers attain adult levels after undergoing a rapid decrease in cell number in the third postnatal week

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Abstract

During postnatal development, microglia, CNS resident innate immune cells, are essential for synaptic pruning, neuronal apoptosis and remodeling. During this period microglia undergo morphological and phenotypic transformations; however, little is known about how microglial number and density is regulated during postnatal CNS development. We found that after an initial increase during the first 14 postnatal days, microglial numbers in mouse brain began declining in the third postnatal week and were reduced by 50% by 6 weeks of age; these “adult” levels were maintained until at least 9 months of age. Microglial CD11b levels increased, whereas CD45 and ER-MP58 declined between P10 and adulthood, consistent with a maturing microglial phenotype. Our data indicate that both increased microglial apoptosis and a decreased proliferative capacity contribute to the developmental reduction in microglial numbers. We found no correlation between developmental reductions in microglial numbers and brain mRNA levels of *Cd200*, *Cx3Cl1*, *M-Csf* or *Il-34*. We tested the ability of *M-Csf*-overexpression, a key growth factor promoting microglial proliferation and survival, to prevent microglial loss in the third postnatal week. Mice overexpressing *M-Csf* in astrocytes had higher numbers of microglia at all ages tested. However, the developmental decline in microglial numbers still occurred, suggesting that chronically elevated M-CSF is unable to overcome the developmental decrease in microglial numbers. Whereas the identity of the factor(s) regulating microglial number and density during development remains to be determined, it is likely that microglia respond to a “maturation” signal since the reduction in microglial numbers coincides with CNS maturation.

Keywords

apoptosis; proliferation; development; flow cytometry; gene expression; M-CSF

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INTRODUCTION

Microglia, the principal resident innate immune cells in the central nervous system (CNS), have diverse roles in health and disease. Microglial activation is associated with virtually all CNS disorders or injuries, although their role in pathophysiology is not fully understood. Emerging studies suggest that microglia also play active roles in the healthy CNS (Tremblay et al. 2011). In the developing brain, microglia are essential for synaptic pruning, developmental neuronal apoptosis and remodeling (Paolicelli et al. 2011; Schafer et al. 2012; Tremblay et al. 2010; Wake et al. 2009); whereas in the adult CNS, microglia are involved in neuroplasticity, maintaining homeostasis and surveillance (Davalos et al. 2005; Kettenmann et al. 2011; Nimmerjahn et al. 2005; Parkhurst et al. 2013).

Contrary to neurons and glia that originate from the neuroectoderm, microglial cells are derived from mesodermal tissue originating in the yolk sac, and they populate the CNS during embryogenesis (Ginhoux et al. 2010; Schulz et al. 2012). Recently, Kierdorf et al. (Kierdorf et al. 2013) identified $CD45^+ c\text{-kit}^0 CX3CR1^-$ cells in the yolk sac as microglial precursors that mature into $CD45^+ c\text{-kit}^- CX3CR1^+$ cells that proliferate and differentiate into microglia in a Pu.1- and Irf8-dependent manner. Although microglia colonize the CNS during embryogenesis before the blood-brain-barrier closes, they retain high mitotic activity during the first two postnatal weeks, resulting in an increased number of these cells in the developing brain (Alliot et al. 1999; Zusso et al. 2012).

Microglia display an activated morphology and have high phagocytic activities during the postnatal period (the first three weeks after birth) (Schwarz et al. 2012). In addition, we have previously shown that microglia express higher levels of *iNOS*, *TNFA* and *Arginase-I* mRNA in early postnatal development compared to the adult CNS (Crain et al. 2013), suggesting that microglial activities in the developing CNS may be distinct from those in the adult. Contrary to the developing brain, microglia in the healthy adult CNS have low mitotic activity (Harry and Kraft 2012) and are characterized by a ramified morphology, with highly motile processes that constantly survey their microenvironment (Nimmerjahn et al. 2005). However, in response to pathogens, injury or pathological processes, microglia become activated, and they can proliferate and migrate to the site of disturbance (Davalos et al. 2005; Kettenmann et al. 2011). Indeed, many CNS disorders are characterized by a several fold increase in microglial cell numbers (Ladeby et al. 2005; Nikodemova et al. 2014).

Thus, microglia have diverse functional roles in the healthy CNS, and they undergo striking transformations in both morphology and activity during development (Harry and Kraft 2012). However, little is known about whether microglial numbers and phenotypes also change during transition from the postnatal period to the adult, or how these changes are regulated. In this study we evaluated the expression of microglial cell surface markers, proliferative/survival signals and microglial numbers and density from postnatal day 3 (P3) to adulthood in the mouse brain. We tested the ability of *M-Csf* overexpression, a potent microglial proliferative/survival stimulus to affect developmental course in microglial numbers using a mouse model in which *M-Csf* was overexpressed in the CNS (De et al. 2014).

METHODS

Animals

Animals were housed in AAALAC-accredited facilities, and all experiments were conducted under protocols approved by the University of Wisconsin Institutional Animal Care and Use Committee. Pregnant or 9 month-old ICR/CD1 mice were purchased from Charles River (Wilmington, MA, USA) and housed under standard conditions (12 hours light/dark cycle, water and food available *ad libitum*). Pups were weaned between 23–25 days of age. Both male and female mice were used in this study. *M-Csf*-overexpressing mice were created on the C57Bl/6J genetic background at the University Wisconsin-Madison as described in detail previously (De et al. 2014). Briefly, TRE-CSF1 mice were crossed with GFAP-tTA mice resulting in GFAP-driven overexpression of *M-Csf* in astrocytes. Littermates lacking one or both transgenes were used as controls.

Microglial isolation

CD11b⁺ cells (microglia) were isolated as we have described in detail previously (Nikodemova and Watters 2012). All reagents were obtained from Miltenyi Biotec (Germany). Briefly, mice ranging in age from 3–270 days were transcardially perfused with cold PBS and brains (including cerebellum and brain stem) were dissected, weighed and enzymatically digested. Myelin was removed by centrifugation in 30% Percoll followed by staining with PE-conjugated CD11b-antibodies. After incubation with anti-PE magnetic beads, microglia were separated in a magnetic field using MS columns. Both CD11b⁺ (microglia) and CD11b[−] fractions (brain homogenates depleted of microglia – subsequently referred to as microglia-free homogenates) were collected and used for further analyses. We previously reported comparable isolation efficiency of cells with both low and high CD11b expression levels using this method (Nikodemova and Watters 2012), so potential age-related changes in CD11b expression should not affect the yield of isolated cells. Microglial yield was determined by counting live cells based on Trypan blue dye exclusion using a hemocytometer. The density of CD11b⁺ cells in the brain is expressed as number of cells/mg tissue. The total number of microglia in adult (P42) *M-Csf*-overexpressing mice was previously reported (De et al. 2014); here, we express these data as microglial density/mg of brain tissue.

RNA isolation and qRT-PCR

RNA isolation and qRT-PCR were performed as we described in detail previously (Crain et al. 2009; Nikodemova and Watters 2011). Total RNA was extracted from isolated CD11b⁺ cells as well as from microglia-free homogenates (see above) using Tri-reagent (Sigma, MO, USA). After cDNA synthesis using a Moloney Murine Leukemia Virus (MMLV) reverse transcriptase (Invitrogen, Grand Island, NY), quantitative PCR was performed using Power SYBR Green Solution (Applied Biosystems, Foster City, CA, USA). Primer sequences are provided in Table 1. Gene expression was normalized to 18s and relative gene expression levels were determined by the $\Delta\Delta C_t$ method (Livak and Schmittgen 2001). Gene expression was considered undetectable if the C_t values were > 35 cycles.

Flow cytometry analysis

Mouse brains were mechanically dissociated using a glass-teflon homogenizer and filtered through 70 μ m cell strainer. Cells were resuspended in permeabilization buffer (PB, PBS supplemented with 0.1% BSA and 0.2% saponin) for 10 min on ice. After centrifugation, cells were stored at -20°C in a modified zinc-based fixative (0.5% zinc chloride, 0.5% zinc trifluoroacetate, 0.05% calcium acetate in 0.1M Tris-HCL, pH to 6.4–6.7) and glycerol (1:1), as previously described (Jensen et al. 2010; Lykidis et al. 2007), until further analysis.

Zinc-fixed cells were washed with PBS, centrifuged and resuspended in PB. Cells were stained for membrane proteins with primary conjugated anti-CD11b-PE (Miltyeni), CD45-APC (BD) and ER-MP58-FITC antibodies (Santa Cruz, CA, USA). Intracellular staining was performed with anti-Ki67 (Chemicon, Billerica, MA) and anti-ssDNA antibodies (Abcam, Cambridge, MA). Secondary PECy7- conjugated antibodies were used to visualize Ki67 (anti-rabbit IgG) and ssDNA (anti-mouse IgM). DAPI (10 $\mu\text{g/ml}$) was added 30 min before samples were analyzed by flow cytometry. To control for non-specific binding, cells were stained with secondary antibodies alone. Flow cytometry analysis was performed using a LSR II (BD Biosciences). All appropriate compensation and fluorescence minus one (FMO) controls were performed and utilized in the analysis, as previously described (Smith et al. 2014). Samples were first gated to exclude doublets and any events off scale with the following singlet gates: FSC-Width/SSC-Area, SSC-Width/FSC-Area. A cell cycle gate was then used to identify cells with intact nuclei based on DAPI-Width/DAPI-Area plotted on a linear scale. Cells were gated based on CD11b⁺ immunoreactivity for post-sort population analyses and graphical representations, which were performed using FlowJo software v.10 (TreeStar Inc.).

Statistical analysis

Results are expressed as the mean $\pm$ SE with $n=3-7$ for each time point. Data were analyzed by one-way ANOVA followed by the Fisher LSD post-hoc test using SigmaStat software (Systat, San Jose, CA, USA). $P<0.05$ was considered significant.

RESULTS

Microglial cell numbers increase within the first 2 postnatal weeks, and decline in the third

CD11b⁺ cells were isolated and counted from the brain tissues of mice ranging in age from 3 to 270 days. We observed a 2.2-fold increase in the total number of CD11b⁺ cells during the first two weeks after birth, followed by a significant decline beginning in the third week (Fig 1A). By 6 weeks of age microglial numbers were reduced by 50% from peak levels at P14, and these numbers were maintained at least until 9 months of age. Brain weight steadily increased up to P14 after which time brain mass only slightly increased until it reached adult levels by 6 weeks of age. The density of microglial cells in the brain (the number of microglia/mg tissue) was significantly higher (by more than 2 times) during postnatal development (P3–P14) than in adulthood (Fig 1B). Microglial density started to decline in the third postnatal week, and by 6 weeks of age and after, there were approximately 2.7 times fewer microglia/mg tissue than in the P3–P14 brain.

Expression of microglial cell surface markers CD11b, CD45 and ER-MP58 changes with age

To begin phenotypic characterization of microglia during development, we analyzed CD11b and CD45 protein levels by flow cytometry during postnatal development. CD11b and CD45 are cell surface proteins important for immune cell function, and expression levels of these markers can be used to distinguish different subsets of immune cells including microglia (CD11b⁺/CD45^{low}) and macrophages (CD11b⁺/CD45^{high}) (Penninger et al. 2001; Schafer et al. 2012; Wakselman et al. 2008). Microglial expression of CD11b was low at P10 and P15 but steadily increased between P22 and P40 (Fig 2A). In contrast, the frequency of CD11b⁺ cells with high levels of CD45 decreased after P10 (Fig 2B, C). However, we detected only a small proportion of brain CD11b⁺ cells that expressed high levels of CD45 at all ages examined, with the largest CD45^{high} population detected at P10 (about 8% of CD11b⁺ cells). The frequency of CD11b⁺/CD45^{high} cells declined by approximately half in postnatal weeks 2, 3 and into adulthood (Fig 2C). These data suggest that microglia in the early postnatal brain are not fully mature, and that they undergo a maturation process in later postnatal development to eventually acquire the adult microglial phenotype (CD11b⁺/CD45^{low}). These observations also suggest the presence of myeloid precursor cells in the brain during early postnatal development. This notion is supported by the higher frequency of CD11b⁺ cells expressing ER-MP58 in the first 2 postnatal weeks compared to the adult CNS (Fig 2D). ER-MP58 is a myeloid cell precursor marker expressed on M-CSF-responsive bone marrow-derived cells (Chan et al. 1998). Although there are no reports of ER-MP58 expression in microglia to our knowledge, we find that ER-MP58 levels decline with age, as microglia mature. This decrease in ER-MP58 protein levels upon microglial maturation is consistent with the decline in ER-MP58 expression observed upon macrophage maturation (Chan et al. 1998).

Microglia have increased proliferative capacity prior to the second postnatal week, whereas apoptotic frequency is increased during the third postnatal week

To examine whether cell proliferation and apoptosis were responsible for the significant increase in microglial number in the first two postnatal weeks and the later decrease in the third postnatal week, respectively, we evaluated microglial cell cycle and apoptosis by flow cytometry at P10, P15, P22 and P40. DNA content measured by DAPI staining was used to distinguish cell cycle phases (Fig 3A) as has been reported previously (Otto 1990). The majority of CD11b⁺ cells (>80%) were in the G₀/G₁ phase at all ages examined. Approximately 10% of CD11b⁺ cells were in the G₂/M phase at P10 and P15, whereas only 5% of CD11b⁺ cells were in the G₂/M phase at P22 and P40 (Fig 3B). Conversely, only a small fraction (~4%) of the CD11b⁺ population was apoptotic during the first two postnatal weeks or at P40. However, at P22 when we observed a significant decline in CD11b⁺ cell number, the frequency of apoptotic cells was almost doubled. Furthermore, in the first 2 postnatal weeks, there were 2.5 times more cells in the proliferative state than in the apoptotic state whereas at P22, the proportion of apoptotic CD11b⁺ cells was 35% higher than the G₂/M phase. Together, these data suggest that the overall population of CD11b⁺ cells in the brain increases within the first two postnatal weeks and that it decreases starting at P22. Interestingly, at P40, the proportion of apoptotic and proliferative CD11b⁺ cells was similar suggesting a stable CD11b⁺ population in the adult CNS. That microglia are

proliferating during early postnatal development is further supported by the analysis of Ki67, a protein associated with cellular proliferation (Fig 3C). We found that 7% of CD11b⁺ cells were also Ki67⁺ at P10, with decreasing expression after this age. In the adult brain (P40), only 1% of microglia were Ki67⁺. Likewise, immunostaining for single-stranded DNA (ssDNA), a marker of apoptosis, was significantly higher at P22 than at any other age evaluated (Fig 3D), strongly supporting the cell cycle results that suggest that microglia undergo apoptosis at this age.

Brain M-CSf and Il-34 levels peak in the third postnatal week and decline by adulthood

To assess the potential regulatory mechanisms controlling developmental changes in microglial number, we evaluated brain expression levels of *M-CSf* and *Il-34*, key growth factors necessary for microglial proliferation and survival (Wang et al. 2012; Wegiel et al. 1998). We reasoned that if they contributed to microglial proliferation during this developmental period, that the expression of these potent proliferative stimuli would be upregulated during the first 2 postnatal weeks, and down-regulated in the third postnatal week, at the time of microglial cell number decline. Surprisingly, the expression of both *M-CSf* (Fig 4A) and *Il-34* (Fig 4B) in microglia-free brain homogenates peaked at P21, although they were also slightly elevated at P14. Their levels were significantly lower in the first postnatal week at P3 and P7, levels which were similar to those in the adult CNS. Microglial expression of the *M-CSf* receptor (*Csf1R*) that mediates microglial responses to both M-CSF and IL-34, was significantly elevated at P14 and P21 (Fig 4C), mirroring the temporal profile of the ligands.

Cd200 and Cx3CII levels peak in the third postnatal week and decline by adulthood

We next examined the expression of *Cd200* and *Cx3CII* in microglia-free brain homogenates because these factors are also important regulators of microglial proliferation and survival (Boehme et al. 2000; Chitnis et al. 2007; Deckert et al. 2006; Lee et al. 2010; Liu et al. 2013; Liu et al. 2014; Tang et al. 2014). *Cd200* expression increased from P3 to P21 whereafter it was down-regulated at P42 (Fig 5A). Decreased *Cd200* expression in the adult CNS is in line with previous reports (Schwarz et al. 2012). The expression of *Cx3CII* was low during the first postnatal week compared to other ages, but by the third postnatal week its expression was increased by 14-fold (Fig 5B). Although *Cx3CII* expression was down-regulated at P42, it remained significantly higher than in the early postnatal period, also consistent with previous reports (Schwarz et al. 2012). The expression of the receptors for *Cd200* (*Cd200r*) and *Cx3CII* (*Cx3CrI*) on microglia seemed to vary less across developmental time points. *Cd200* was elevated by 1.5-fold at P14 compared to P3 (Fig 5C), and *Cx3CrI* expression was increased in microglia at both P14 and P42 (Fig 5D), almost exactly inversely paralleling *Cx3CII* levels.

M-CSF overexpression in astrocytes does not prevent the developmental reduction in microglial cell number

Since insufficient CSF1R signaling contributes to the loss of microglial cells in the adult (De et al. 2014; Elmore et al. 2014), we hypothesized that chronically elevated M-CSF levels in the CNS during development could prevent the natural developmental decline in microglial

number. Thus, we explored the functional role of M-CSF in the developmental regulation of microglial number using mice overexpressing *M-Csf* in astrocytes under control of the *GFAP* promoter on the C57Bl/6J genetic background. As expected, we found that the levels of *M-Csf* mRNA in microglia-free brain homogenates were significantly higher in the *M-Csf*-overexpressing mice than control mice (Fig 6A) at both P14 and P42, whereas the expression of *Il-34* was not different (Fig 6B). We previously showed that these mice have ~2.5 times more microglia in the adult CNS (P42) compared to littermate controls (De et al. 2014); Fig 6C), and that continued signaling through the CSF1R was necessary for microglial survival in the adult brain. Furthermore, we show here that *M-Csf*-overexpressing mice at P14 also had 33% more microglia than control mice (Fig 6C). Interestingly, at P42 we observed a significant decrease in microglial numbers in both control and *M-Csf*-overexpressing mice, indicating that chronically elevated levels of M-CSF in the brain could not prevent the decline in microglial number that occurs during postnatal development. However, it appears that the 48% developmental decline in microglial number in *M-Csf*-overexpressing mice was significantly less than the 69% decrease observed in control mice ($p < 0.001$), indicating that *M-Csf* overexpression may partially protect microglia from undergoing apoptosis during postnatal development. There were no statistically significant differences in microglial numbers in the control mice on the C57Bl/6J genetic background (Fig 6C) and ICR/CD1 (Fig 1), indicating that these observations are not simply due to strain differences.

Because *M-Csf*-overexpression only partially prevented the developmental down-regulation of microglial number, and CD200 and CX3CL1 are important regulators of microglial proliferation and/or survival both *in vitro* and *in vivo* (Boehme et al. 2000; Chitnis et al. 2007; Deckert et al. 2006; Lee et al. 2010; Liu et al. 2013; Liu et al. 2014; Tang et al. 2014), we also examined their expression in the *M-Csf*-overexpressing mice at P14 and P42. The normal developmental down-regulation of *Cd200* expression levels that occurs between P14 and P42 in wild-type mice does not occur in *M-Csf*-overexpressing mice (Fig 6D). However, *Cx3cl1* mRNA levels were significantly increased in the *M-Csf*-overexpressing mice at both ages (Fig 6E), suggesting that *CX3CL1* upregulation by M-CSF may play a role in promoting microglial survival in these mice.

DISCUSSION

In this study, we describe the developmental course of microglial number in the brain. The total number of microglia in brain parenchyma increases during the first two postnatal weeks as has been previously reported (Dalmau et al. 2003; Harry and Kraft 2012), reaching a peak around 14 days of age. However, in the third postnatal week, microglial numbers begin declining, and by 6 weeks of age, total brain microglial numbers are 2 times lower compared to their peak levels at 14 days age, numbers which are subsequently maintained throughout adulthood. We observe that this regulated decline in microglial number involves increased apoptosis and decreased proliferation. That the density of microglia in the developing CNS is two times higher than in the adult CNS spawns several important questions: what is the need for the higher microglial density in the developing CNS; what are the mechanisms responsible for the reduction in microglial number; and what are the signals initiating and regulating this developmental reduction?

Microglia exist in different functional states in the postnatal and the adult CNS. During the postnatal period, microglia have an amoeboid morphology and high phagocytic activity (Schwarz et al. 2012), and we recently showed a significant up-regulation of some pro-inflammatory genes in postnatal microglia, although they did not acquire the typical M1 phenotype (Crain et al. 2013). In the present study, we provide further evidence that microglia undergo their own developmental course. In the adult brain, microglia typically express CD11b and low levels of CD45 (Nikodemova and Watters 2012), distinguishing them from macrophages that are characterized by high CD11b and CD45 expression (Sedgwick et al. 1991). In the postnatal brain, the majority of microglia express low levels of CD11b and CD45. However, a small proportion (approximately 8%) of the early postnatal CNS CD11b⁺ population expressed high levels of CD45 and ER-MP58, suggesting that these cells may be myeloid precursors, the frequency of which gradually diminished until adulthood. Our data indicate that these putative precursors may have a higher proliferative capacity than microglia, since almost 20% of the CD11b⁺/CD45^{high} cells were in the G2/M cell cycle phase at P10, whereas only 5% of CD11b⁺/CD45^{low} cells were in the G2/M phase (data not shown).

Two mechanisms may be responsible for the observed developmental reductions in microglial number: decreased proliferation and/or increased apoptosis. Our data suggest that both mechanisms may play a role in this process. However, we cannot exclude the possibilities that some portion of microglia may lose CD11b expression or leave the CNS, although such precedents have not been reported. We find that at P22, the proportion of apoptotic microglial cells is increased, whereas the proportion of cells in the G2/M phase is decreased. The increased levels of ssDNA immunoreactivity at P21 further support the conclusion that microglia undergo developmental apoptosis. Thus, together with the decreased frequency of cells in the proliferative state, we predict that the overall population of CD11b⁺ cells would decrease at this time point. We did not evaluate microglial number between the third and sixth week of age, so the precise timing of when the brain attains the “adult” microglial density remains to be determined. In the adult CNS, microglia are long-lived and have a very slow turn-over rate (Lawson et al. 1992; Saijo and Glass 2011), and our data also support this observation. We found that in the adult brain, only a small percentage of CD11b⁺ cells (around 4%) are in the G2/M phase as assayed by DNA content, and less than 2% express Ki67, a nuclear protein associated with proliferation, consistent with previous reports using autoradiography (Imamoto and Leblond 1978). In addition, we found that the frequency of microglial cells in the apoptotic state was similar to the number in the proliferative state, enabling the maintenance of a stable population in the adult CNS.

The processes regulating microglial number in the CNS are unknown. Mice lacking the receptor for macrophage-colony stimulating factor (CSF1R) are severely depleted of microglia (Erblich et al. 2011), suggesting that this receptor is essential for microglial development. Both M-CSF and IL-34, ligands for the CSF1R, are highly expressed in the CNS (Berezovskaya et al. 1995; Nandi et al. 2012; Wang et al. 2012). M-CSF-deficient *op/op* mice and IL-34-deficient mice have significantly decreased numbers of microglia in the CNS (Kondo and Duncan 2009; Wang et al. 2012). On the other hand, mice overexpressing M-CSf in GFAP⁺ cells have increased numbers of microglia (De et al. 2014).

Therefore, we hypothesized that decreased CNS availability of the CSF1R ligands, M-CSF and IL-34, in the third postnatal week would play a key role in promoting microglial apoptosis. Unexpectedly, our data showed a significant up-regulation of both *M-Csf* and *Il-34* between P14 and P21, accompanied by a transient up-regulation of *Csf1R* on microglia. We also found that microglial numbers still declined between P14 and adulthood in *M-Csf*-overexpressing mice, suggesting that alterations in other factors may be responsible for decreasing microglial proliferation and/or promoting their apoptosis during normal postnatal brain development. However, since the reduction in microglial numbers in *M-Csf*-overexpressors was significantly smaller than in control mice (48% vs 69%, respectively), we hypothesize that the upregulation of M-CSF and/or IL-34 that occurs in the normal (wild-type) CNS at P21 may be a protective mechanism to prevent larger scale microglial apoptosis during this time. More studies are needed to understand the significance of the developmental decrease in microglial number for the adult CNS. We recently showed that adult microglia had impaired responses to systemic inflammation in *M-Csf*-overexpressing mice where microglial numbers were 2–3 times too high (De et al. 2014), indicating that improper regulation of microglial number may be detrimental. However, it is not clear at this time if these compromised microglial responses to inflammation result from the abnormally high microglial numbers, or from the elevated expression of brain *M-Csf* that may independently influence microglial immune responses. In addition, although *M-Csf*-overexpressing mice appear outwardly normal, they have not been subjected to any neurologic testing.

The timing of developmental reduction of microglial number coincides with previously reported morphologic changes in microglia from an amoeboid, activated state, to a ramified, quiescent phenotype (Schwarz et al. 2012). In the adult brain, the quiescent state of microglia is maintained by CD200 and CX3CL1. Both are membrane proteins mostly expressed by neurons (Chitnis et al. 2007; Eyo and Wu 2013), that regulate microglial proliferation and/or survival *in vivo* and *in vitro* (Boehme et al. 2000; Chitnis et al. 2007; Deckert et al. 2006; Lee et al. 2010; Liu et al. 2013; Liu et al. 2014; Tang et al. 2014). We found significantly elevated expression levels of *Cd200* and *Cx3cl1* in the brain at P14 and P21 correlating with the time of microglial phenotypic and morphologic transition. However, it is not yet clear whether the increased expression of these factors plays any role in the developmental regulation of microglial numbers, although mice lacking *Cx3cr1* (the receptor for CX3CL1), have slightly reduced microglial numbers between postnatal days 8 and 28 (Paolicelli et al. 2011).

In conclusion, we found that microglial cell numbers in the CNS are developmentally regulated. Microglial density in the postnatal CNS is two times higher than in the adult, indicating that increased apoptosis and/or decreased proliferation may play a significant role in regulating microglial numbers at key postnatal developmental times. Because the majority of synaptic pruning, neuronal circuit refinement and myelination occurs during the first three postnatal weeks of mouse brain development (Hashimoto and Kano 2013; Verma et al. 2005), microglia play a vital role in all of these processes, and the reduction in microglial number coincides with CNS maturation, we hypothesize that there are “maturation” signals that regulate microglial number which are responsible for this developmental decrease. The

differences in the density and phenotypes of microglia between the postnatal and adult CNS support the notion that during the early postnatal period, the primary function of microglia may be to participate in “building” the CNS, whereas in adulthood, microglia switch to a maintenance/surveillance mode. These studies set the stage for needed mechanistic investigations into cell-cell communications that instruct microglial proliferation and apoptosis during critical postnatal developmental periods.

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Main points

After the second postnatal week, microglial numbers decline by 50% to reach adult levels by 6 weeks of age. Concomitant increases in apoptosis and decreases in proliferation contribute to tight regulation of microglial numbers in the developing CNS.

Highlights

- Microglia proliferate within the first 2 postnatal weeks
- Microglial numbers decline by 50% between the third and sixth postnatal weeks
- Increased apoptosis and decreased proliferation contribute to tight regulation
- Brain M-CSF-overexpression does not prevent developmental microglial density decline

Fig. 1. Microglial cell numbers increase within the first two postnatal weeks, and decline in the third. Microglia were immunomagnetically isolated from ICR/CD1 mouse brains ranging from 3 to 270 days of age. (A) Age-related changes in the total number of microglia isolated and in brain weights. (B) Microglial brain density expressed as the number of microglia per mg tissue. n= 3–10 per time point; **p<0.01, ***P<0.001 vs 3 days of age.

Fig. 2.

Microglial cell surface CD11b, CD45 and ER-MP58 marker expression levels mature postnatally. Single cell suspensions prepared from the brains of 10, 15, 22 and 40 day-old ICR/CD1 mice were stained with CD11b, CD45 and ER-MP58 antibodies, and analyzed by flow cytometry. Cells were gated based on CD11b⁺ immunoreactivity. (A) Histogram of CD11b expression on microglia at different ages. (B) CD11b and CD45 expression in 10 and 40 day-old mouse brain. The horizontal line divides cells possessing low (below line) and high (above line) CD45 immunoreactivity. (C) Percentage of CD11b⁺ cells expressing high levels of CD45. (D) Percentage of CD11b⁺ cells expressing the myeloid precursor marker ER-MP58. $n=3$; * $p<0.05$ vs 10 days of age, ** $p<0.01$ vs 10 days of age.

Fig. 3.

Microglia have increased proliferative capacity prior to the second postnatal week, whereas apoptotic frequency is increased during the third postnatal week. Single cell suspensions prepared from the brains of 10, 15, 22 and 40 day-old ICR/CD1 mice were stained with CD11b, Ki67 and ssDNA antibodies and DAPI followed by flow cytometry analysis. Cells were gated based on CD11b⁺ immunoreactivity. (A) Dot plot of CD11b⁺/CD45⁺ cells in different phases of the cell cycle based on DAPI staining. (B) Proportion of cells (expressed as % of total CD11b⁺ cells) in G2/M and apoptotic phases of the cell cycle at different ages based on DAPI staining. (C) Frequency of CD11b⁺ cells expressing Ki67 at different ages. (D) Mean fluorescent intensity (MFI) of ssDNA staining in CD11b⁺ cells. *p<0.05 vs respective phase at 10 days of age, **p<0.01 vs respective phase at 10 days of age, ***p<0.001 vs 10 days of age.

Fig. 4.

Brain *M-CSf* and *Il-34* mRNA levels peak in the third postnatal week and decline by adulthood. Total RNA was isolated from CD11b⁺ cells and microglia-free brain homogenates, and analyzed by qRT-PCR. *M-CSf* (A) and *Il-34* (B) expression levels in microglia-free brain homogenates from 3 day- to 6 week-old ICR/CD1 mice. (C) *Csf1R* expression in microglia isolated from brains of 3 day- to 6 week-old ICR/CD1 mice. n=3; *p<0.05, ***p<0.001 vs 3 days of age.

Fig. 5.

CD200 and *CX3CL1* levels peak in the third postnatal week and decline by adulthood. Total RNA was isolated from CD11b⁺ cells and microglia-free brain homogenates and analyzed by qRT-PCR. *CD200* (A) and *CX3CL1* (B) expression levels in microglia-free brain homogenates from 3 day- to 6 week-old ICR/CD1 mice. Expression of corresponding receptors *CD200R* (C) and *CX3CR1* (D) on microglia isolated from the brains of 3 day- to 6 week-old ICR/CD1 mice. n=3; *p<0.05, **p<0.01, ***p<0.001 vs 3 days of age.

Fig. 6.

M-Csf-overexpression in astrocytes does not prevent the developmental reduction in microglial cell number. Microglia were immunomagnetically isolated from brains of C57Bl/6J control mice or mice overexpressing *M-Csf* under the control of the *GFAP* promoter (*M-Csf* overexpressors). Total RNA was isolated from microglia-free brain homogenates and analyzed by qRT-PCR. *M-Csf* (A), *Il-34* (B), *Cd200* (D) and *Cx3C1* (E) expression levels in 14 and 42 day-old mice. (C) Number of CD11b⁺ cells, expressed as the number of cells/mg tissue, isolated from 14 and 42 day-old mice. $n=4-6$ (A, B, D, E), $n=6-10$ (C); ** $p<0.01$, *** $p<0.001$ *M-Csf*-overexpressors vs control of the same age; ## $p<0.01$, ### $p<0.001$ age difference (P42 vs P14) on the same genetic background.

Table 1

Primer Sequences

	Forward Sequence- 5' → 3'	Reverse Sequence- 5' → 3'
M-CSF	AACACCCCAATGCTAACGCCA	ACACAGGCCTTGTCTGCTCC
IL-34	TACAGCGGAGCCTCATGGATGT	ATGACCCGGAAGCAGTTGTCCA
CSF-1R	TGGCCACAGTTTGGCATGGTCA	ACACATCGCAGGGTCACCGTTT
CD200	CACAGCTCAAGTGGAAGTGGTG	TTCTGCCATGTCACAATCAAGG
CD200R	AGTGAGCGGCGGAAAACCAGAA	AACTTGACCCAGCCACAAAGACCC
CX3CL1	ACCTCACGAATCCCAGTGGCTT	TCTCCAGGACAATGGCACGCTT
CX3CR1	TGCTTGACATTGGGGAGACTGGA	AGGGAACGCTAAAGTCCTGGCTGA
18s	CGGGTGCTCTTAGCTGAGTGTCCCG	CTCGGGCCTGCTTTGAACAC