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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.

1. 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. (2013) identified $CD45^+$ c-kit^{lo} $CX3CR1^-$ cells in the yolk sac as microglial precursors that mature into $CD45^+$ c-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*, *TNFα* 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

☆ *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.

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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 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).

2. Methods

2.1. 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 hour light/dark cycle, water and food available ad libitum). Pups were weaned between 23 and 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 of 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.

2.2. 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 to 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.

2.3. 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 18 s and relative gene expression levels were determined by the ΔΔCt method (Livak and Schmittgen, 2001). Gene expression was considered undetectable if the Ct values were >35 cycles.

Table 1
Primer sequences.

	Forward sequence- 5′ → 3′	Reverse sequence- 5′ → 3′
M-CSF	AACACCCCCAATGCTAACGCCA	ACACAGGCCTTGTTCTGCTCC
IL-34	TACAGCGGAGCCTCATGGATGT	ATGACCCGGAAGCAGTTGTCCA
CSF-1R	TGGCCACAGTTTGGCATGGTCA	ACACATCGCAGGGTCACCGTTT
CD200	CACAGCTCAAGTGGAAGTGGTG	TTCTGCCATGTCACAATCAAGG
CD200R	AGTGAGCGGCGGAAAACCAGAA	AACTTGACCCAGCCACAAAGACCC
CX3CL1	ACCTCACGAATCCCAGTGGCTT	TCTCCAGGACAATGGCACGCTT
CX3CR1	TGCTTGCACATTGGGGAGACTGGA	AGGGAACGCTAAAGTCCTGGCTGA
18s	CGGGTGCTCTTAGCTGAGTGTCCCG	CTCGGGCCTGCTTTGAACAC

2.4. Flow cytometry analysis

Mouse brains were mechanically dissociated using a glass-teflon homogenizer and filtered through 70 μm 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 0.1 M Tris–HCl,

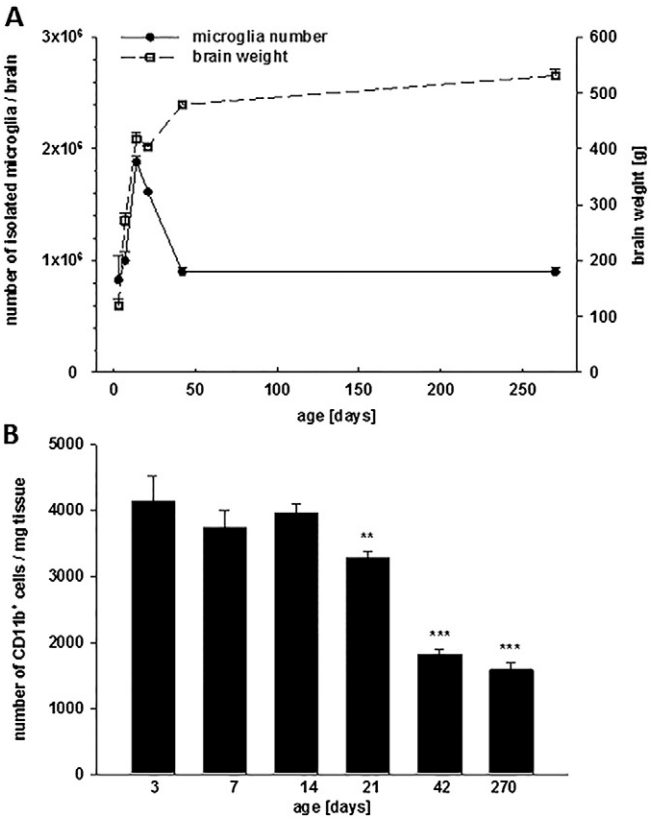

Fig. 1. Microglial cell numbers increase within the first two postnatal weeks, and decline in the third. Microglia were immunomagnetically isolated from ICR/CD1 mouse brains ranging from 3 to 270 days of age. (A) Age-related changes in the total number of microglia isolated and in brain weights. (B) 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.