Published in final edited form as:

Brain Behav Immun. 2016 July ; 55: 49–59. doi:10.1016/j.bbi.2015.11.009.

Developmental Changes in Microglial Mobilization are Independent of Apoptosis in the Neonatal Mouse Hippocampus

Ukpong B. Eyo*, Samuel A. Miner, Joshua A. Weiner, and Michael E. Dailey

Department of Biology, University of Iowa, Iowa City, IA 52242

Abstract

During CNS development, microglia transform from highly mobile amoeboid-like cells to primitive ramified forms and, finally, to highly branched but relatively stationary cells in maturity. The factors that control developmental changes in microglia are largely unknown. Because microglia detect and clear apoptotic cells, developmental changes in microglia may be controlled by neuronal apoptosis. Here, we assessed the extent to which microglial cell density, morphology, motility, and migration are regulated by developmental apoptosis, focusing on the first postnatal week in the mouse hippocampus when the density of apoptotic bodies peaks at postnatal day 4 and declines sharply thereafter. Analysis of microglial form and distribution *in situ* over the first postnatal week showed that, although there was little change in the number of primary microglial branches, microglial cell density increased significantly, and microglia were often seen near or engulfing apoptotic bodies. Time-lapse imaging in hippocampal slices harvested at different times over the first postnatal week showed differences in microglial motility and migration that correlated with the density of apoptotic bodies. The extent to which these changes in microglia are driven by developmental neuronal apoptosis was assessed in tissues from BAX null mice lacking apoptosis. We found that apoptosis can lead to local microglial accumulation near apoptotic neurons in the pyramidal cell body layer but, unexpectedly, loss of apoptosis did not alter overall microglial cell density *in vivo* or microglial motility and migration in *ex vivo* tissue slices. These results demonstrate that developmental changes in microglial form, distribution, motility, and migration occur essentially normally in the absence of developmental apoptosis, indicating that factors other than neuronal apoptosis regulate these features of microglial development.

Keywords

microglia; motility; migration; mobility; apoptosis

Correspondence: Dr. Michael Dailey, Dept. of Biology, 369 Biology Building, University of Iowa, Iowa City, IA 52242-1324, TEL: (319) 335-1067, FAX: (319) 335-1069, michael-e-dailey@uiowa.edu.

*Current address: Department of Cell Biology and Neuroscience, Rutgers University, Piscataway, NJ 08854

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

1. INTRODUCTION

Microglia are immunocompetent cells of the central nervous system (CNS). Though once thought to be quiescent or “resting” cells in the uninjured brain, *in vivo* imaging has shown that microglia in the adult brain are extremely motile (constantly remodeling their branch projections) though non-migratory (i.e. without soma translocation) (Davalos et al. 2005; Li et al. 2012; Nimmerjahn et al. 2005; Wake et al. 2009), leading to the recognition that microglia are “surveying”, rather than “resting,” cells (Hanisch and Kettenmann 2007). However, less is known about their motility and migration in the developing brain and no studies in mammals currently exist to describe microglial movements *in vivo* during such periods even though microglia are proposed to play significant roles during development (Eyo and Dailey 2013; Pont-Lezica et al. 2011; Schafer et al. 2013; Schlegelmilch et al. 2011)

In the current study, we determined microglial dynamics during the first week of postnatal hippocampal development in the mouse. Having observed a peak of apoptotic cell debris at P4 in the CA1 region of the hippocampus, we speculated that apoptosis might regulate microglial morphological development and dynamics. First, we describe the increasing density and structural heterogeneity of microglia during this period (P2 – P6). Subsequently, microglial mobilization (which we define as including both soma migration and process motility) were monitored during *ex vivo* time-lapse imaging in freshly excised tissue slices. We show significant changes in microglial process motility and migration over this period: while microglial mobilization remains high through P4, it falls significantly by P6, and this change correlates with the changes in developmental apoptosis *in vivo*.

Given that: (i) microglia accumulate in areas of developmental apoptosis; (ii) high microglial mobilization *ex vivo* correlated with peak periods of developmental apoptosis *in vivo*, and (iii) declining mobilization *ex vivo* correlated with declining apoptosis *in vivo*, we tested the hypothesis that developmental apoptosis regulates microglial (a) entry and/or maintenance (b) mobilization *ex vivo* and (c) distribution during hippocampal murine development. To do this, we compared microglial mobilization in wild type and BAX knockout littermate mice at P4 when developmental apoptotic debris is maximal in area CA1. Despite the lack of apoptosis, there was no significant difference in microglial density *in vivo* or microglial mobilization *ex vivo* with BAX deficiency. Moreover, although we observed that microglial accumulation in the neuronal cell body layer was significantly reduced in BAX knockouts at P4, this returned to normal by P9. Our results indicate that microglial entry, maintenance, and mobilization occur independent of BAX-regulated apoptosis, although apoptosis can lead to local microglial accumulation in the *stratum pyramidale* of early postnatal hippocampus that is restored after apoptotic debris is cleared.

2. MATERIALS AND METHODS

2.1. Animals and Preparation of Tissue Slices

Reporter mice expressing GFP under the control of the fractalkine receptor (CX3CR1) promoter (Jung et al. 2000) were obtained from The Jackson Laboratory (Bar Harbor, ME) and used for all experiments. Only heterozygous CX3CR1^{+/GFP} mice were used in these

experiments to avoid any phenotypes due to CX3CR1 deficiency; though none have been observed, we cannot completely rule out possible subtle effects on microglial mobility and chemotaxis in these CX3CR1^{+/GFP} heterozygous mice. In these mice, GFP is expressed in parenchymal microglia, as well as in perivascular cells and meningeal cells that are easily distinguishable from parenchymal microglia in the brain. For some experiments, BAX null mice (Knudson et al., 1995) were crossed with CX3CR1 GFP reporter mice to generate BAX wildtype (BAX^{+/+}:CX3CR1^{+/GFP}) and BAX knockout (BAX^{-/-}:CX3CR1^{+/GFP}) littermates. Acutely isolated hippocampal slices were prepared from neonatal (P2–P6 unless otherwise stated) mice as detailed previously (Eyo and Dailey 2012). Briefly, mice were swiftly decapitated, and brains were removed and placed in ice-cold artificial cerebrospinal fluid (ACSF) with the following composition (in mM): NaCl 124; KCl 3; NaH₂PO₄ 1.3; MgCl₂ 3; HEPES 10; CaCl₂ 3; glucose 10. Excised hippocampi were cut transversely (400 μ m thick) using a manual tissue chopper (Stoelting). Slices were maintained in HEPES-buffered ACSF. Animals were used in accordance with institutional guidelines, as approved by the animal care and use committee.

2.2. Time-Lapse Confocal Imaging

Acutely excised tissue slices were mounted in a custom-built closed chamber containing ~3 mL HEPES-buffered ACSF. The chamber was then placed on the microscope stage and warmed to ~35°C by continuous, gentle warm air (Dailey et al., 2011, 2013). Fluorescence images were captured using a Leica SP5 MP confocal/multiphoton imaging system with a xyz motorized stage on an upright platform. For confocal microscopy, the following probes were imaged with the indicated laser lines: GFP (Argon 488 nm), Sytox Orange (HeNe 543 nm), PSVue-550 (HeNe 543 nm) or PSVue-647 (HeNe 633 nm). The confocal pinhole typically was opened to two Airy disc units to improve light collection and increase signal-to-noise ratio (Dailey et al., 2006). The chamber media was not changed during the course of imaging as previous experiments showed no significant effect of a media change for neonatal slices over this period of imaging (unpublished data). To capture a large (775 μ m $\times$ 775 μ m) field of view, images were collected using a 20 $\times$ /0.7 Plan Apo objective lens at a resolution of 1.4 pixels/ μ m. A typical time-lapse imaging session captured 15 confocal optical planes at 3 μ m z-step intervals spanning 45–60 μ m in the axial (z) dimension from the slice surface. Stacks of confocal images were usually captured at 10 min intervals. For all experiments, multisite imaging of several slices was employed. In some cases, this allowed us to image tissue slices from separate littermate animals simultaneously under identical conditions. Imaging sessions typically commenced about 30 min after tissue slicing and lasted three hours.

2.3. Image Processing

Images were collected and collated using Leica LAS AF software. Image stacks were assembled using Leica LAS AF software or ImageJ (Wayne Rasband, NIH). All images were processed using the “Smooth” filter in ImageJ to reduce noise. In all cases, comparisons were made on images processed identically. All movies generated represent the same xyz tissue volume size, although they may differ in lengths of time.

2.4. Analysis of Microglial Motility

We used an automated approach to measure microglial cell motility in ImageJ (Eyo and Dailey, 2012). First, 3D image stacks were combined to make 2D projection images for each time-point. Next, to account for any x-y tissue drift during the imaging session, 2D projection images were registered using the StackReg plugin (Gonzales and Crews, 1985) running in ImageJ. Registered images were then smoothed to reduce background noise. To define the cell boundary, an arbitrary threshold was applied uniformly to all images in a given time sequence. To generate difference images, the absolute difference between two sequential thresholded images in a time series was calculated using the 'Difference' tool of the 'Image Calculator' feature of ImageJ. Sequential difference images in a time sequence were used to generate a motility index (MI), which is a percent change in area calculated as follows: $MI = (\text{Area of difference between adjacent images} / \text{Total suprathreshold area of first time point}) \times 100$. MI was used for both single cell and multiple cell analyses.

2.5 Analysis of Microglial Migration

Two types of migration analyses were performed. Blind analysis was done on timelapse movies in which all cells were analyzed and tracked automatically. To define cell bodies in movies, images were thresholded (at a grayscale value of 200) and used for tracking with the MTrack2 plugin in ImageJ (Tarnawski et al. 2013). Object size was set to 50–1000 pixels. The maximum velocity was set at 60 units and the minimum track length was 50 minutes. For a second type of analysis, only the most migratory cells were tracked manually using the MTrackJ plugin of ImageJ and the velocity and distance travelled were quantified. These cells were selected subjectively as the most actively migrating (based on the distance travelled) cells in the field of view per movie. Only cells that were present all through the period of imaging were selected for analysis as some cells migrated out of the field of view.

2.6. Quantification of Microglia and PSVue Density

Acutely excised tissue slices were immediately placed in 3.7% paraformaldehyde/PBS fix for 1 hour. Tissues were then washed three times with PBS and incubated in a solution containing the late apoptotic cell dye PSVue 550 (1:500 in PBS; Molecular Targeting Technologies, Inc.) for 2 hours at room temperature, followed by PBS wash three times (Ahlers et al., 2015). Tissues were then mounted and imaged by confocal microscopy to generate 45 μm deep image stacks (3 μm z-step intervals). GFP positive microglia and PSVue positive structures in hippocampal area CA1 were manually counted in confocal image stacks, the area of the whole CA1 was determined using ImageJ, and tissue volume was determined by multiplying area by the tissue depth (45 μm). Microglia and PSVue particle density was then calculated as: number of microglia or PSVue structure/tissue volume.

2.7. Statistical Analysis

Data from several slices and multiple mice were pooled and analyzed. Each mouse represents a separate experiment and at least three mice were used for each set of experiments. All results are reported as mean $\pm$ standard error of the mean (SEM). For all analyses, statistical significance was assessed using Student's t-test. For multiple

comparison analysis between P2, P4, and P6, we performed multiple Student's t-tests with Bonferroni corrections, then multiplied the calculated p value obtained for each comparison by the number of comparisons in order to keep the reported levels of significance the same throughout the manuscript. For Fig. 3I, the Mann-Whitney U test was used for analysis of primary process number. We used Microsoft Excel for all t-test analyses and the Mann Whitney U calculator from the following website: <http://www.socscistatistics.com/tests/mannwhitney/>. For all statistics, significance was ascertained at the significance level P 0.05.

3. RESULTS

3.1. Developmental Apoptosis in the Early Postnatal Mouse Hippocampus *In Situ*

To investigate the regulation of microglial morphology and dynamics during the first postnatal week in the CA1 region of the murine hippocampus, we first examined developmental apoptosis in freshly prepared live or fixed tissue slices from neonatal mice using PSVue, a fluorescent marker that labels later-stage apoptotic bodies (Ahlers et al. 2015). Since apoptotic signals have been reported to attract phagocytic cells (Elliott et al. 2009) and developmental apoptosis in the hippocampus drops sharply during the first postnatal week (Murase et al. 2011), we speculated that developmental apoptosis may alter microglial distribution and behavior over this time period. We performed staining for apoptotic cell debris in mouse tissues expressing GFP under the control of the fractalkine receptor promoter [CX3CR1^{GFP/+}; (Jung et al. 2000)] at P2 (Fig. 1A–C), P4 (Fig. 1D–F), and P6 (Fig. 1G–I). Microglia were frequently found engulfing PSVue⁺ structures (Fig. 1B, E, H), or were in close proximity to non-engulfed PSVue structures (Fig. 1C, F, I). Qualitative observations (Fig. 1A, D, G and Supplemental Movie 1) suggested that the highest level of apoptotic bodies occurred at P4, and this was confirmed by quantitative analysis of PSVue density (Fig. 1J). In the *stratum pyramidale* or *stratum radiatum*, we sometimes observed linear arrays of PSVue-labelled structures that we surmised were remnants of degenerating neuronal dendrites (Fig. 1K–L). Our observations indicate that neuronal apoptosis in area CA1 of the postnatal murine hippocampus *in vivo* peaks around P4.

3.2. Microglial Cell Density Increases During the First Postnatal Week in the Mouse Hippocampus

Signals from dying cells may regulate microglial distribution, morphology, and mobilization during the early postnatal period of development so we investigated microglial density during this period. In acutely excised and fixed hippocampal slices from GFP reporter mice, we found that microglial cell density increased during this time (Fig. 2A–C). Quantitative analysis showed that microglial cell density increased significantly from P2 ($5.7 \pm 0.7 \times 10^{-6}$ cells/ μm^3) to P4 ($6.6 \pm 0.7 \times 10^{-6}$ cells/ μm^3) and from P4 to P6 ($9.7 \pm 1.4 \times 10^{-6}$ cells/ μm^3) (Fig. 2D). Although rare, we observed microglial cell division during *ex vivo* time-lapse imaging of live neonatal hippocampal slices. One such event is presented in Fig. 2E where the parent cell rounds up and rapidly divides into two daughter cells that subsequently extend branches and resume motile activity (See also Supplemental Movie 2). This indicates

that local mitotic activity may contribute to the increase in microglial cell density during early postnatal development in the hippocampus.

3.3. Maintenance of Microglial Structural Diversity During the First Postnatal Week in the Mouse Hippocampus

Next, we quantified microglial morphology in area CA1 at P2, P4, and P6. As reported previously in rats (Dalmau et al. 1997; Dalmau et al. 1998), primitive ramified microglia (PRM) were observed in the developing hippocampus by P2 (the earliest time we studied) and continued through P6. PRM usually had one to six primary projections (Fig. 3A–G). Quantitative analysis in six acutely excised hippocampal slices from three animals at each age indicated that the majority of microglia (65.4% at P2; 59.3% at P4; and 61.2% at P6) possessed two or three primary projections. These structural features did not change significantly during the first postnatal week (Fig. 3H, I).

3.4. *Ex Vivo* Mobilization Changes Significantly During the First Postnatal Week

To study microglial mobilization in real-time during early postnatal development, we performed confocal time-lapse imaging *ex vivo* in acutely excised hippocampal slices. Here, we distinguished two aspects of microglial mobilization: i) microglial *motility*, defined as cell process movements assessed by a motility index assay [see Methods and (Eyo and Dailey 2012)] and ii) microglial *migration* defined as cell body translocation and assessed by tracking of microglial cell body movements through time in time-lapse movies. Consistent with our previous reports (Eyo and Dailey 2012; Kurpius et al. 2007), we found that the branches of microglia in neonatal slices are highly motile. Using a previously described motility index (MI) assay (Eyo and Dailey 2012), we found microglial motility to be high at P2 (110 ± 1.2) and P4 (124 ± 1.6) but sharply reduced by P6 (68 ± 0.7) (Fig. 4A–C and Supplemental Movie 3).

The motility index detects changes in microglial cell area, which includes both the microglial cell body and processes. However, time-lapse imaging indicates that, unlike in acutely isolated adult tissues (Carbonell et al. 2005), microglia in neonatal tissues are not stationary but migratory (Stence et al. 2001; Grossmann et al. 2002; Kurpius et al., 2006). Thus, to determine the extent to which migratory behavior of tissue microglia may change across the first postnatal week, we quantified microglial migration in time-lapse movies in neonatal slices from P2 to P6 mice. Using an automated approach to analyze microglial cell migration on a population basis, we found that the average rate of migration of the population as a whole was significantly higher at P2 (0.60 ± 0.02) and P4 (0.59 ± 0.03) relative to P6 (0.28 ± 0.01) (Fig. 5B).

In our time-lapse movies we noticed that migration rates varied widely among microglia. Although at the population level migration rates were similar at P2 and P4, our impression was that the most migratory microglia moved faster in P4 tissues. Thus, we extended our analysis to include manual tracking of the five most migratory cells in each movie (see Methods) as a measure of the peak migration ability at these ages (Fig. 5A). The average velocity in these cells at P2 was $1.2 \pm 0.07 \mu\text{m}/\text{min}$, and this increased significantly to $1.7 \pm 0.13 \mu\text{m}/\text{min}$ at P4 and then dropped significantly to $0.6 \pm 0.03 \mu\text{m}/\text{min}$ at P6 (Fig. 5C).

Similarly, the peak instantaneous velocity at P2 was $3.6 \pm 0.4 \mu\text{m}/\text{min}$, which increased significantly at P4 ($5.7 \pm 0.4 \mu\text{m}/\text{min}$) and then dropped at P6 ($1.5 \pm 0.14 \mu\text{m}/\text{min}$) (Fig. 5D). Together, these analyses of microglial migration at both the population and individual cell levels show that *ex vivo* microglial migration is high during early postnatal development but declines sharply between P4 and P6 (See also Supplemental Movie 4).

3.5. BAX Deficiency Transiently Alters Microglial Distribution but not Overall Density in the Developing Hippocampal Area CA1

Thus far our data indicate a correlation between the peak of developmental apoptosis *in vivo* and microglial mobilization in age-matched acutely excised *ex vivo* tissue slices, leading us to speculate that apoptotic cell death may regulate microglial cell density and mobilization. To test this idea, we crossed mice lacking the gene for BAX, a key component of the pro-apoptotic machinery (Deckwerth et al. 1996; White et al. 1998), with the CX3CR1 GFP-reporter line to generate BAX wild type and knockout littermates that express GFP in microglia in the brain parenchyma. As reported previously (Ahlers et al. 2015), PSVue staining was evident in neonatal brain tissues from wild type mice but not from BAX knockouts (Fig. 6A–C), indicating that PSVue labels apoptotic structures. Despite the strong effect of BAX KO on developmental apoptosis in BAX KO mice, we found that microglial density in hippocampal area CA1 was not significantly different in tissues from BAX wild type ($6.96 \pm 0.36 \times 10^{-6} \text{ cells}/\mu\text{m}^3$) and BAX knockout mice ($6.6 \pm 0.24 \times 10^{-6} \text{ cells}/\mu\text{m}^3$) at P4 (Fig. 6D–F), a time when the density of apoptotic cell debris is normally highest.

In assessing the distribution of apoptotic cells and microglia in neonatal mouse hippocampus, we noticed that PSVue-positive apoptotic bodies had accumulated in the CA1 *stratum pyramidale* (SP) in wild type mice, and microglia also were evident in the SP in wild type mice but rarely in BAX KO mice. Quantitative analysis showed significantly reduced microglial cell density in the SP in BAX knockouts ($6.5 \pm 0.2 \times 10^{-6} \text{ cells}/\mu\text{m}^3$) when compared to wild type littermates at P4 ($9.8 \pm 0.3 \times 10^{-6} \text{ cells}/\mu\text{m}^3$) (Fig. 7A–C). However, this difference was transient because by P9, when developmental apoptosis in CA1 is essentially complete, microglial density in the CA1 SP was identical in BAX wild type and knockout tissues (Fig. 7D–F). This result was not due to differences between wild type and BAX null mice in the whole area CA1 ($7.8 \pm 0.4 \times 10^{-6} \mu\text{m}^3$ in wild type and $7.9 \pm 0.2 \mu\text{m}^3$ in BAX KO mice; $P = 0.6$) or of the SP ($1.3 \pm 0.04 \times 10^{-5} \mu\text{m}^3$ in wild type and $1.4 \pm 0.1 \times 10^{-5} \mu\text{m}^3$ in BAX KO mice; $P = 0.6$). Together, these data indicate that developmental neuronal apoptosis is not required for overall microglial colonization (entry and/or proliferation) of the developing hippocampus, but it does regulate the local density of microglia within the *stratum pyramidale* in the developing hippocampus.

3.6. BAX Deficiency Does Not Alter *Ex Vivo* Microglial Mobilization in Tissue Slices from the Developing Hippocampus

Finally, we performed time-lapse imaging of GFP-expressing microglia in acutely excised tissue slices harvested from BAX wild type and knockout mouse littermates at P4, a time of peak accumulation of apoptotic bodies (Fig. 8). Population analysis indicated no differences in migration between microglia from wild type (0.62 ± 0.07) and BAX knockout (0.61 ± 0.02) littermates (Fig. 8A, B). Similarly, selective analysis of the most migratory cells

showed that BAX deficiency did not alter the total distance travelled by parenchymal microglia at P4 (Fig. 8C). Moreover, microglia from both genotypes displayed indistinguishable motility indices (Fig. 8D; See also Supplemental Movie 5), suggesting that apoptotic cell death during early postnatal development *in vivo* is not responsible for differences in microglial mobilization within the hippocampus.

4. DISCUSSION

The major findings of this study are: (1) an increase in the accumulation of apoptotic cell debris (PSVue) in the developing hippocampus that peaks at ~P4 and drops significantly by P6; (2) an increase in microglial cell density and maintenance of morphological heterogeneity during early postnatal hippocampal development; (3) a rapid mobilization of microglia *ex vivo* including cell soma migration and branch process motility that remains high up to P4 but declines sharply by P6; (4) a transient increase in microglial density near apoptotic pyramidal neuron cell bodies within the early postnatal hippocampus and (5) microglial colonization and *ex vivo* mobilization are independent of developmental cell death in the hippocampus.

4.1. Transient Accumulation of Apoptotic Bodies During Early Postnatal Hippocampal Development

Apoptotic cell death is a widespread phenomenon in the developing brain (Burek and Oppenheim 1996). Like previous studies (Ahlers et al. 2015; Ferrer et al. 1990; Mallat et al. 2005), we found that microglia were frequently located close to and/or engulfing apoptotic bodies. Moreover, we observed a peak in apoptotic cell debris in the hippocampus at P4 with a significant drop by P6 (Fig. 1). This finding is consistent with a previous study (Murase et al. 2011), which reported a peak in hippocampal apoptosis (assessed by cleaved caspase 3 immunoreactivity) at P3 with a significant drop by P7. Although another study (Wakselman et al. 2008) observed peak apoptosis in the hippocampus occurring a few days earlier at P0–1, that study was performed in the subicular complex, while our study focused on the CA1 region. Given recent interest in microglial activity during development (Bilimoria and Stevens 2014; Eyo and Dailey 2013; Michell-Robinson et al. 2015; Pont-Lezica et al. 2011; Schafer et al. 2013; Schlegelmilch et al. 2011), these observations during the first postnatal week in the hippocampus identify a good time window to study changes in the functional activity of microglia (especially phagocytosis) during peak periods of developmental apoptosis.

Recent *in vivo* imaging in the developing zebrafish showed remarkably that apoptotic cells are rarely visualized outside of microglia, suggesting that microglia rapidly engulf and clear apoptotic debris (Peri and Nusslein-Volhard 2008; Svahn et al. 2013). Moreover, in the young adult mouse, microglia efficiently phagocytized apoptotic newborn cells (Sierra et al. 2010). Our observations in the neonatal mouse brain differ somewhat from these previous studies because we found many apoptotic bodies outside of microglia in the hippocampus (this study, Fig. 1 and Supplemental Movie 1) and in the neocortex (Ahlers et al. 2015). One potential explanation for this difference is that PSVue may label a larger set of apoptotic structures than markers used in other studies. Regardless, our data indicate that the rate of

apoptotic cell death during mouse brain development exceeds the capacity of microglia to immediately clear the debris.

4.2. Microglial Cell Density During Early Postnatal Development

We observed a progressive increase in microglial cell density in hippocampal area CA1 during the first postnatal week of development (Fig. 2), and this increase continued at least up to P15 (data not shown) which is also consistent with recent reports of increasing microglial number up until the third week of postnatal development in the mouse (Nikodemova et al. 2015). Previous studies in the rat showed a similar increase in total microglial cells in the postnatal hippocampus despite the fact that there were no significant changes in cell density (Dalmau et al. 2003). The discrepancy between that study and ours could be accounted for by species differences (rat versus mouse).

The increase in microglial cells in the developing hippocampus may result from infiltration of microglial precursors and/or microglial cell proliferation. Dalmau et al. (2003) reported increased microglial labeling for a proliferation marker, PCNA, from late embryonic stages through the first two postnatal weeks, and this peaked between P6 and P9 when 95% of microglia were PCNA-positive. Similar to the observations in the rat brain (Dalmau et al. 2003), *in vivo* imaging in the zebrafish brain detected microglial proliferation during early development (Herbomel et al. 2001; Svahn et al. 2013). However, in our time-lapse imaging studies in excised tissue slices, mitotic microglia were rarely observed during the usual three hour imaging period and required longer periods of imaging for detection (e.g., Fig. 2E).

Although it is possible that perturbation of the native hippocampal environment and/or imaging procedures may inhibit microglial proliferation, we have observed dozens of mitotic microglia in neonatal rat hippocampal slice cultures by the same time-lapse imaging methodology used here (Petersen and Dailey, 2004). Moreover, despite widespread cell proliferation (BrdU uptake) in the developing mouse brain, microglial proliferation in the neurogenic niche during the first postnatal week was reported to be very low (Xavier et al. 2015). It remains to be determined whether this also holds true for hippocampal microglia during the first postnatal week *in vivo*. Alternatively, the increase in microglial cells during this developmental period may largely result from continual migration into the hippocampus rather than proliferation of resident microglia.

4.3. Microglial Morphology During Early Postnatal Development

Earlier descriptions of microglia (Giulian and Baker 1986; Ling 1976) indicated that microglial precursors in the developing brain are amoeboid in morphology and progressively increase their ramification into adulthood (Kettenmann et al. 2011). So-called primitive ramified microglia (PRM) begin to appear in the prenatal and early postnatal rat hippocampus (Dalmau et al. 1997; Dalmau et al. 1998). Recently, PRM were shown to co-exist with amoeboid microglia in the mouse embryonic spinal cord as early as E12.5, and by E15.5 all microglia were considered PRM (Rigato et al. 2011). In the early neonatal murine hippocampus, we found that in categorizing microglial morphology by the number of primary processes, microglial morphology was relatively stable during the first postnatal week (Mann-Whitney U test *P* values between 0.07 and 0.87), and most PRM possessed 2 or

3 primary processes (Fig. 3). Nevertheless, we cannot exclude the possibility of differences in secondary and tertiary branching during early postnatal development in the mouse hippocampus. Moreover, molecular heterogeneity of microglia was not assessed.

4.4. Microglial Mobilization During Early Postnatal Development

One of the more intriguing findings of this study is the capability of early postnatal microglia to rapidly migrate. Previous studies have reported high rates of microglial migration in tissue slices. Amoeboid microglia in acutely isolated corpus callosum tissue slices from neonatal (P5–9) mice were observed to migrate at 0.5 – 1 $\mu\text{m}/\text{min}$ (Brockhaus et al. 1996). Rates of microglial migration in our P6 mouse hippocampal slices are similar (0.5 – 1.5 $\mu\text{m}/\text{min}$). We previously observed that microglia in P4 rat hippocampal slices migrate at up to 2 $\mu\text{m}/\text{min}$ (Stence et al. 2001). Here, we describe microglia that migrate at peak speeds of almost 6 $\mu\text{m}/\text{min}$ during the first few days after birth (Fig. 5). The higher rates of migration at early stages of development may enable microglia to more effectively patrol brain tissues and clear apoptotic debris during peak periods of apoptotic cell death.

Equally remarkable is the dramatic reduction in both motility and migration between P4 and P6 (Figs. 4 and 5). Although it is possible that this is due to a sharp decline in viability of cells and tissues between P4 and P6, we note that *ex vivo* imaging in the embryonic mouse cortex shows a similar rapid decrease in microglial migration at a different developmental stage [between E14.5 and E17.5; (Swinnen et al. 2013)]. This indicates that the rapid decline in microglial migration is not tightly tied to age of the animal but may be more closely related to region-specific changes in brain development. However, it remains to be determined whether the speeds observed in *in vitro* and *ex vivo* preparations of the neonatal rodent brain are representative of the condition *in vivo* because, to our knowledge, there are no published studies describing microglial mobilization in the intact neonatal brain.

On this point, there is a disparity between *in vivo* and *ex vivo* studies of microglial migration because migration is not observed in the adult mouse brain *in vivo* for several hours after injury (Davalos et al. 2005; Nimmerjahn et al. 2005), whereas there is considerable microglial migration in tissue slices from neonates (Brockhaus et al. 1996; Kurpius et al. 2006; Petersen and Dailey 2004; Stence et al. 2001). These differences may be due to differences in tissue preparations (*ex vivo* versus *in vivo*) or to differences in ages (neonates versus adults). Indeed, a caveat of the current approach is that microglial motility and migration may be affected by the tissue excision. Although technically difficult to perform, *in vivo* studies in neonates are needed to resolve this issue.

Although it is possible that acute tissue injury during slice preparation may induce the migration of neonatal microglia in *ex vivo* slices, it is also likely that neonatal microglia in the mammalian brain have a higher intrinsic capacity for migration. First, microglial migration is not observed in freshly excised brain tissue slices from adult mice even in the presence of slice-induced injury, indicating that tissue injury alone during slice preparation is not sufficient to induce rapid microglial migration in acute brain slices (Carbonell et al. 2005; Eyo et al. 2014; Wu et al. 2007). In this respect, it is important to note that adult microglia can migrate in response to injury *in vivo* (Kim and Dustin 2006) and in tissue slices freshly prepared 1–3 days after injury *in vivo* (Carbonell et al. 2005).

Observations in the developing zebrafish also indicate high migratory capacities for developing microglia in uninjured conditions. *In vivo* imaging during the 3rd and 4th days post-fertilization revealed that microglia are not stationary but migratory (Sieger et al. 2012), and a subsequent study (Svahn et al. 2013) reported that the migration of zebrafish microglia peaks at 4 days after fertilization and drops significantly a day later. It remains to be determined whether the rapid decline in microglial migration observed during development in both fish and mouse is due to cell-autonomous changes in the microglial capacity for migration or to non-cell-autonomous changes within developing brain tissues.

4.5. BAX-Deficiency and Microglia during Early Postnatal Development

BAX is a pro-apoptotic protein whose role in the regulation of apoptosis during development has been clearly established (Deckwerth et al. 1996; Knudson et al. 1995). While the effect of BAX-deficiency in neuronal death has been extensively studied, its effect on microglial accumulation and mobility in the brain has not been reported. Dalmau et al. (2003) observed some TUNEL positive microglia in the P6 and P9 rat hippocampus, suggesting that some microglia may die by apoptosis during postnatal development in rats. In our study, we found that BAX-deficiency did not alter overall microglial cell density in the hippocampal area CA1 at P4 when apoptosis is maximal (Fig. 6) or at P15 (data not shown), suggesting that during early postnatal development, there is no significant die-off of microglia by BAX-dependent apoptosis. This is consistent with a previous study in the P4 rat optic nerve concluding that the bulk of apoptotic cell death in non-neuronal cells is not prevented by deletion of BAX (White et al. 1998).

Given that microglia normally accumulated near apoptotic cells, we were surprised that loss of apoptosis in BAX KO mice did not alter microglial cell density or *ex vivo* mobilization in hippocampal tissues. Given this observation, we do not know what factor(s) regulate(s) baseline motility and migration, or the rapid reduction in microglial mobility observed between P4 and P6. Perhaps microglial mobility is more directly regulated by developmental changes in synaptic function, including changes in neurotransmitter or gliotransmitter signaling. Because purines have been shown to strongly regulate microglial motility and migration (Madry and Attwell 2015; Ohsawa and Kohsaka 2011) these molecules are good candidates for regulating developmental changes in microglial behavior. However, microglia in P2Y₁₂ receptor KO mice retain basal motility (Haynes et al. 2006), indicating that other signaling pathways must regulate these features of microglial behavior. Regardless, our observations here indicate that apoptotic signals are not the primary mediators of the changes in microglial motility and migration observed during postnatal development.

Despite the lack of an effect on microglial motility and migration in *ex vivo* time-lapse studies, we found that BAX deficiency inhibits microglial accumulation in the pyramidal cell body layer at P4 when apoptosis is maximal *in vivo* (Fig. 7), suggesting that apoptotic signals can indeed alter the local distribution of microglia. Such effects on microglia distribution may be transient, however, because hippocampal microglia redistribute after clearance of apoptotic cell debris, much as they do after ethanol-induced apoptotic cell death in neonatal mouse neocortex (Ahlers et al. 2015). Although it seems likely that microglia are

transiently attracted to the *SP* by apoptotic pyramidal neurons, other factors may also directly or indirectly regulate the distribution of microglia during development.

5. CONCLUSIONS

Here we have described developmental changes in microglial mobilization in tissues from the early postnatal mouse hippocampus. Microglia in the adult brain have been described as “surveilling” cells because they continually scan the nervous tissue with their elaborately ramified processes while keeping their cell soma stationary (Hanisch and Kettenmann 2007; Nimmerjahn et al. 2005). Our observations during the first postnatal week in the mouse hippocampus, together with the observations of others in the zebrafish optic tectum (Sieger et al. 2012; Svahn et al. 2013) and the embryonic mouse spinal cord (Rigato et al. 2011), suggest that microglia in the developing CNS may be properly termed “patrolling” microglia because both their processes and somata are dynamic. As microglial cell density increases and apoptotic cell death decreases during development, “patrolling” microglia may gradually transform into “surveilling” microglia. Both “patrolling” and “surveilling” microglia may function to effectively scan the nervous tissue and help maintain homeostasis. It is possible that migration may be advantageous for effective tissue surveillance at early stages in development when microglial cell density is low. As microglial density increases, microglial process activity may be sufficient to enable adequate tissue surveillance, reducing the necessity for migration. At any rate, the capacity for microglia to rapidly mobilize in response to brain tissue injury appears to be correlated with peak periods of apoptotic cell death, although BAX-dependent apoptotic cell death does not appear to be a principal driver for these changes in microglia. The factors that control these developmental changes remain to be discovered.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

ROLE OF THE FUNDING SOURCE

Supported by grants to M.E.D. from the NIH (NS043468, AA018823), the American Heart Association (0950160G), the UI Biological Sciences Funding Program, and the Iowa Center for Molecular Auditory Neuroscience through NIH Grant P30 DC010362 (S. Green, PI).

References

- Ahlers KE, Karacay B, Fuller L, Bonthius DJ, Dailey ME. Transient activation of microglia following acute alcohol exposure in developing mouse neocortex is primarily driven by BAX-dependent neurodegeneration. *Glia*. 2015 Apr 9;doi: 10.1002/glia.22835
- Bilimoria PM, Stevens B. Microglia function during brain development: New insights from animal models. *Brain Res*. 2014; 1617:7–17. [PubMed: 25463024]
- Brockhaus J, Moller T, Kettenmann H. Phagocytosing amoeboid microglial cells studied in a mouse corpus callosum slice preparation. *Glia*. 1996; 16:81–90. [PubMed: 8787776]
- Burek MJ, Oppenheim RW. Programmed cell death in the developing nervous system. *Brain Pathol*. 1996; 6:427–46. [PubMed: 8944315]

- Carbonell WS, Murase S, Horwitz AF, Mandell JW. Migration of perilesional microglia after focal brain injury and modulation by CC chemokine receptor 5: an in situ time-lapse confocal imaging study. *J Neurosci*. 2005; 25:7040–7. [PubMed: 16049180]
- Dailey, ME.; Manders, E.; Soll, D.; Terasaki, M. Confocal microscopy of living cells. In: Pawley, JB., editor. *Handbook of Biological Confocal Microscopy*. 3. New York: Springer; 2006. p. 381–403.
- Dailey, ME.; Marrs, GS.; Kurpius, D. Maintaining live cells and tissue slices in the imaging setup. Chapter 33. *Imaging in Neuroscience: A Laboratory Manual*. In: Helmchen, F.; Konnerth, A., editors. Cold Spring Harb Protoc. 2011 Apr 1. pdb.top105
- Dailey ME, Eyo U, Fuller L, Hass J, Kurpius D. Imaging microglia in brain slices and slice cultures. *Cold Spring Harb Protoc*. 2013 Dec 2.;1142–1148. [PubMed: 24298036]
- Dalmau I, Finsen B, Tonder N, Zimmer J, Gonzalez B, Castellano B. Development of microglia in the prenatal rat hippocampus. *J Comp Neurol*. 1997; 377:70–84. [PubMed: 8986873]
- Dalmau I, Finsen B, Zimmer J, Gonzalez B, Castellano B. Development of microglia in the postnatal rat hippocampus. *Hippocampus*. 1998; 8:458–74. [PubMed: 9825958]
- Dalmau I, Vela JM, Gonzalez B, Finsen B, Castellano B. Dynamics of microglia in the developing rat brain. *J Comp Neurol*. 2003; 458:144–57. [PubMed: 12596255]
- Davalos D, Grutzendler J, Yang G, Kim JV, Zuo Y, Jung S, Littman DR, Dustin ML, Gan WB. ATP mediates rapid microglial response to local brain injury in vivo. *Nat Neurosci*. 2005; 8:752–8. [PubMed: 15895084]
- Deckwerth TL, Elliott JL, Knudson CM, Johnson EM Jr, Snider WD, Korsmeyer SJ. BAX is required for neuronal death after trophic factor deprivation and during development. *Neuron*. 1996; 17:401–11. [PubMed: 8816704]
- Elliott MR, Cheken FB, Trampont PC, Lazarowski ER, Kadl A, Walk SF, Park D, Woodson RI, Ostankovich M, Sharma P, et al. Nucleotides released by apoptotic cells act as a find-me signal to promote phagocytic clearance. *Nature*. 2009; 461:282–6. [PubMed: 19741708]
- Eyo U, Dailey ME. Effects of oxygen-glucose deprivation on microglial mobility and viability in developing mouse hippocampal tissues. *Glia*. 2012; 60:1747–60. [PubMed: 22847985]
- Eyo UB, Dailey ME. Microglia: key elements in neural development, plasticity, and pathology. *J Neuroimmune Pharmacol*. 2013; 8:494–509. [PubMed: 23354784]
- Eyo UB, Peng J, Swiatkowski P, Mukherjee A, Bispo A, Wu LJ. Neuronal Hyperactivity Recruits Microglial Processes via Neuronal NMDA Receptors and Microglial P2Y12 Receptors after Status Epilepticus. *J Neurosci*. 2014; 34:10528–40. [PubMed: 25100587]
- Ferrer I, Bernet E, Soriano E, del Rio T, Fonseca M. Naturally occurring cell death in the cerebral cortex of the rat and removal of dead cells by transitory phagocytes. *Neuroscience*. 1990; 39:451–8. [PubMed: 2087266]
- Giulian D, Baker TJ. Characterization of ameboid microglia isolated from developing mammalian brain. *J Neurosci*. 1986; 6:2163–78. [PubMed: 3018187]
- Hanisch UK, Kettenmann H. Microglia: active sensor and versatile effector cells in the normal and pathologic brain. *Nat Neurosci*. 2007; 10:1387–94. [PubMed: 17965659]
- Haynes SE, Hollopeter G, Yang G, Kurpius D, Dailey ME, Gan WB, Julius D. The P2Y12 receptor regulates microglial activation by extracellular nucleotides. *Nat Neurosci*. 2006; 9:1512–9. [PubMed: 17115040]
- Herbomel P, Thisse B, Thisse C. Zebrafish early macrophages colonize cephalic mesenchyme and developing brain, retina, and epidermis through a M-CSF receptor-dependent invasive process. *Dev Biol*. 2001; 238:274–88. [PubMed: 11784010]
- Jung S, Aliberti J, Graemmel P, Sunshine MJ, Kreutzberg GW, Sher A, Littman DR. Analysis of fractalkine receptor CX(3)CR1 function by targeted deletion and green fluorescent protein reporter gene insertion. *Mol Cell Biol*. 2000; 20:4106–14. [PubMed: 10805752]
- Kettenmann H, Hanisch UK, Noda M, Verkhratsky A. Physiology of microglia. *Physiol Rev*. 2011; 91:461–553. [PubMed: 21527731]
- Kim JV, Dustin ML. Innate response to focal necrotic injury inside the blood-brain barrier. *J Immunol*. 2006; 177:5269–77. [PubMed: 17015712]
- Knudson CM, Tung KS, Tourtellotte WG, Brown GA, Korsmeyer SJ. Bax-deficient mice with lymphoid hyperplasia and male germ cell death. *Science*. 1995; 270:96–9. [PubMed: 7569956]

- Kurpius D, Nolley EP, Dailey ME. Purines induce directed migration and rapid homing of microglia to injured pyramidal neurons in developing hippocampus. *Glia*. 2007; 55:873–84. [PubMed: 17405148]
- Kurpius D, Wilson N, Fuller L, Hoffman A, Dailey ME. Early activation, motility, and homing of neonatal microglia to injured neurons does not require protein synthesis. *Glia*. 2006; 54:58–70. [PubMed: 16715500]
- Li Y, Du XF, Liu CS, Wen ZL, Du JL. Reciprocal regulation between resting microglial dynamics and neuronal activity in vivo. *Dev Cell*. 2012; 23:1189–202. [PubMed: 23201120]
- Ling EA. Some aspects of amoeboid microglia in the corpus callosum and neighbouring regions of neonatal rats. *J Anat*. 1976; 121:29–45. [PubMed: 1254530]
- Madry C, Attwell D. Receptors, ion channels, and signaling mechanisms underlying microglial dynamics. *J Biol Chem*. 2015; 290:12443–50. [PubMed: 25855789]
- Mallat M, Marin-Teva JL, Cheret C. Phagocytosis in the developing CNS: more than clearing the corpses. *Curr Opin Neurobiol*. 2005; 15:101–7. [PubMed: 15721751]
- Michell-Robinson MA, Touil H, Healy LM, Owen DR, Durafour BA, Bar-Or A, Antel JP, Moore CS. Roles of microglia in brain development, tissue maintenance and repair. *Brain*. 2015; 138:1138–59. [PubMed: 25823474]
- Murase S, Poser SW, Joseph J, McKay RD. p53 controls neuronal death in the CA3 region of the newborn mouse hippocampus. *Eur J Neurosci*. 2011; 34:374–81. [PubMed: 21714817]
- Nikodemova M, Kimyon RS, De I, Small AL, Collier LS, Watters JJ. Microglial numbers attain adult levels after undergoing a rapid decrease in cell number in the third postnatal week. *J Neuroimmunol*. 2015; 278:280–8. [PubMed: 25468773]
- Nimmerjahn A, Kirchhoff F, Helmchen F. Resting microglial cells are highly dynamic surveillants of brain parenchyma in vivo. *Science*. 2005; 308:1314–8. [PubMed: 15831717]
- Ohsawa K, Kohsaka S. Dynamic motility of microglia: purinergic modulation of microglial movement in the normal and pathological brain. *Glia*. 2011; 59:1793–9. [PubMed: 21901756]
- Peri F, Nusslein-Volhard C. Live imaging of neuronal degradation by microglia reveals a role for v0-ATPase a1 in phagosomal fusion in vivo. *Cell*. 2008; 133:916–27. [PubMed: 18510934]
- Petersen MA, Dailey ME. Diverse microglial motility behaviors during clearance of dead cells in hippocampal slices. *Glia*. 2004; 46:195–206. [PubMed: 15042586]
- Pont-Lezica L, Bechade C, Belarief-Cantaut Y, Pascual O, Bessis A. Physiological roles of microglia during development. *J Neurochem*. 2011; 119:901–8. [PubMed: 21951310]
- Rigato C, Buckinx R, Le-Corrone H, Rigo JM, Legendre P. Pattern of invasion of the embryonic mouse spinal cord by microglial cells at the time of the onset of functional neuronal networks. *Glia*. 2011; 59:675–95. [PubMed: 21305616]
- Schafer DP, Lehrman EK, Stevens B. The “quad-partite” synapse: microglia-synapse interactions in the developing and mature CNS. *Glia*. 2013; 61:24–36. [PubMed: 22829357]
- Schlegelmilch T, Henke K, Peri F. Microglia in the developing brain: from immunity to behaviour. *Curr Opin Neurobiol*. 2011; 21:5–10. [PubMed: 20817438]
- Sieger D, Moritz C, Ziegenhals T, Prykhodzhiy S, Peri F. Long-range Ca²⁺ waves transmit brain-damage signals to microglia. *Dev Cell*. 2012; 22:1138–48. [PubMed: 22632801]
- Sierra A, Encinas JM, Deudero JJ, Chancey JH, Enikolopov G, Overstreet-Wadiche LS, Tsirka SE, Maletic-Savatic M. Microglia shape adult hippocampal neurogenesis through apoptosis-coupled phagocytosis. *Cell Stem Cell*. 2010; 7:483–95. [PubMed: 20887954]
- Stence N, Waite M, Dailey ME. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia*. 2001; 33:256–66. [PubMed: 11241743]
- Svahn AJ, Graeber MB, Ellett F, Lieschke GJ, Rinkwitz S, Bennett MR, Becker TS. Development of ramified microglia from early macrophages in the zebrafish optic tectum. *Dev Neurobiol*. 2013; 73:60–71. [PubMed: 22648905]
- Swinnen N, Smolders S, Avila A, Notelaers K, Paesen R, Ameloot M, Brone B, Legendre P, Rigo JM. Complex invasion pattern of the cerebral cortex by microglial cells during development of the mouse embryo. *Glia*. 2013; 61:150–63. [PubMed: 23001583]

- Tarnawski W, Kurtcuoglu V, Lorek P, Bodych M, Rotter J, Muszkieta M, Piwowar L, Poulikakos D, Majkowski M, Ferrari A. A robust algorithm for segmenting and tracking clustered cells in time-lapse fluorescent microscopy. *IEEE J Biomed Health Inform.* 2013; 17:862–9. [PubMed: 25055315]
- Wake H, Moorhouse AJ, Jinno S, Kohsaka S, Nabekura J. Resting microglia directly monitor the functional state of synapses in vivo and determine the fate of ischemic terminals. *J Neurosci.* 2009; 29:3974–80. [PubMed: 19339593]
- Wakselman S, Bechade C, Roumier A, Bernard D, Triller A, Bessis A. Developmental neuronal death in hippocampus requires the microglial CD11b integrin and DAP12 immunoreceptor. *J Neurosci.* 2008; 28:8138–43. [PubMed: 18685038]
- White FA, Keller-Peck CR, Knudson CM, Korsmeyer SJ, Snider WD. Widespread elimination of naturally occurring neuronal death in Bax-deficient mice. *J Neurosci.* 1998; 18:1428–39. [PubMed: 9454852]
- Wu LJ, Vadakkan KI, Zhuo M. ATP-induced chemotaxis of microglial processes requires P2Y receptor-activated initiation of outward potassium currents. *Glia.* 2007; 55:810–21. [PubMed: 17357150]
- Xavier AL, Lima FR, Nedergaard M, Menezes JR. Ontogeny of CX3CR1-EGFP expressing cells unveil microglia as an integral component of the postnatal subventricular zone. *Front Cell Neurosci.* 2015; 9:37. [PubMed: 25741237]

Highlights

- Microglia are surveilling cells involved in clearance of apoptotic cells.
- In mouse hippocampus, the density of apoptotic bodies peaks around postnatal day 4.
- Rates of microglial motility and migration correlate with levels of apoptotic debris.
- Yet, loss of apoptosis does not alter microglial cell density, motility or migration.
- Factors other than apoptosis regulate changes in microglial motility and migration.

Figure 1. The Density of Apoptotic Bodies Peaks at P4 in the Neonatal Mouse Hippocampus
A–I, Representative fields of view showing GFP+ microglia (green) and PSVue structures (red) at P2 (**A–C**), P4 (**D–F**), and P6 (**G–I**) in the CA1 region of the hippocampus in tissues from GFP-reporter mice. PSVue structures were usually concentrated in or near the *stratum pyramidale* (SP). Microglia in boxed regions are shown at higher magnification either engulfing nearby PSVue structures (**B**, **E** and **H**) or in close proximity to them (**C**, **F** and **I**). **J**, Quantitative analysis revealed that the density of PSVue structures peaked at P4 then declined afterward. **K–L**, PSVue structures were sometimes found in radially-oriented linear arrays (arrowheads in **L**) consistent with fragmentation of a neuronal dendrite. See also Supplemental Movie 1. ***P < 0.001.

Figure 2. Microglial Cell Density Increases Over the First Postnatal Week in the Developing Mouse Hippocampus

A–C, Representative fields of view showing microglia in the *stratum radiatum* of area CA1 at P2 (A), P4 (B) and P6 (C). D, Microglial density increases significantly from P2 to P4, and from P4 to P6. E, Time-lapse imaging in a live, acutely excised hippocampal slice from P4 mouse shows mitotic division of a microglial cell (yellow arrows). Time shown is in hr:min from the commencement of imaging. See also Supplemental Movie 2. *** $P < 0.001$.

Figure 3. Microglial Branching Is Not Significantly Altered Over the First Postnatal Week in the Developing Hippocampus

A, A representative image from a hippocampal slice at P4 showing the distribution of primitive ramified microglia in the *stratum radiatum* of hippocampal area CA1. Select cells are highlighted in boxed regions in panels B–G. **B–G**, Microglia with one (**B**), two (**C**), three (**D**), four (**E**) five (**F**) or six (**G**) primary processes. **H–I**, Distribution of primitive ramified microglia at postnatal day P2, P4, and P6 showing that most microglia during early postnatal development in the CA1 have 2 or 3 primary processes. There were no significant differences in number of primary processes between these ages. $n = 18$ slices (6 slices from 3 animals) for each age.

Figure 4. Changes in Microglial Motility in Hippocampal Tissue Slices From Neonatal Mice
A, Representative fields of view of difference images from P2, P4, and P6 mice. White areas represent regions of cells that have changed from one time-point to the next (time interval of 10 minutes). **B–C**, Microglial motility index is highest at P4 and drops precipitously at P6. *** $P < 0.001$. $n = 8$ fields of view from 4 slices (2 fields of view per slice) for each age. See also Supplemental Movie 3.

Figure 5. Changes in Microglial Migration in Hippocampal Tissue Slices from Neonatal Mice

A, Representative microglial cells with migration tracks (red lines) during three hours of imaging in tissue slices from P2 (**left panel**), P4 (**middle panel**) or P6 (**right panel**) mice.

B, Quantitative analysis of the total microglial population shows that the average velocity of microglia migration is similar at P2 and P4 but decreases significantly between P4 and P6.

C–D, Single cell analysis of the most migratory cells shows that both the average velocity (**C**) and peak instantaneous velocity (**D**) are highest at P4 and significantly drop by P6. * $P < 0.05$; *** $P < 0.001$. $n = 5$ slices at P2; $n = 5$ slices at P4; and $n = 4$ slices at P6 for **B–D**. See also Supplemental Movie 4.

Figure 6. BAX-Deficiency Abolishes Developmental Apoptosis Without Reducing Overall Microglial Cell Density in Area CA1 at P4

A, B: Representative images in the CA1 region of the hippocampus showing PSVue-labeled apoptotic bodies (white arrowheads) in BAX wildtype (**A**) but not in BAX knockout (**B**) littermate mice at P4. **C,** Quantitative analysis confirms that BAX deficiency significantly reduces developmental apoptosis as detected by PSVue density. **D, E:** Corresponding images of the same field of view in (**A**) and (**B**) showing microglial cell density in slices from BAX wildtype (**D**) and BAX knockout (**E**) littermates at P4. **F,** Despite the significant reduction in apoptotic bodies, BAX deficiency does not significantly alter overall microglial cell density in hippocampal area CA1. ***P < 0.001.

Figure 7. BAX Deficiency Transiently Alters Microglial Accumulation in the CA1 Pyramidal Cell Body Layer (SP)

A–C, Representative images in the CA1 region of the hippocampus of BAX wild type (**A**) and BAX knockout (**B**) slices in GFP-reporter mice at P4. Microglial density is significantly reduced in the *SP* layer of area CA1 in BAX knockout compared to wildtype tissues (**C**). **D–F**, Representative images of BAX wild type (**D**) and knockout (**E**) slices at P9 show high overall microglial cell density, although microglial density is not significantly different in the *SP* layer of knockout compared to wild type mice (**F**). *** $P < 0.001$.

Figure 8. BAX Deficiency Does not Alter Microglial Mobilization in Hippocampal Tissue Slices from P4 Mice

A, Representative images from time-lapse movies collected simultaneously in tissue slices derived from BAX wildtype (left) and BAX knockout (right) mice showing microglia with migration tracks over 3 h (red lines). **B–C**, BAX deficiency did not alter the mean velocity of the microglia population (**B**) or the total distance travelled by the most active microglia (**C**). $n = 9$ slices per genotype in **B** and $n = 5$ slices per genotype in **C** (the 5 most active cells from each slice were analyzed). **D**, Microglial motility index (**D**) is indistinguishable between genotypes. $n = 8$ fields of view from 4 slices. See also Supplemental Movie 5.