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Developmental changes in microglial mobilization are independent of apoptosis in the neonatal mouse hippocampus

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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.

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

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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; NaH_2PO_4 1.3; $MgCl_2$ 3; HEPES 10; $CaCl_2$ 3; glucose 10. Excised hippocampi were cut transversely (400 μm 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 μm × 775 μm) field of view, images were collected using a 20×/0.7 Plan Apo objective lens at a resolution of 1.4 pixels/μm. A typical time-lapse imaging session captured 15 confocal optical planes at 3 μm *z*-step intervals spanning 45–60 μm 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 timepoint. Next, to account for any *x*–*y* tissue drift during the imaging session, 2D projection images were registered using the StackReg plugin (Thevenaz et al., 1998) running in ImageJ. Registered images were then smoothened 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 = (Area of difference between adjacent images/Total suprathreshold area of first time point) × 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 min. 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 traveled were quantified. These cells were selected subjectively as the most actively migrating (based on the distance traveled) 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 h 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

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