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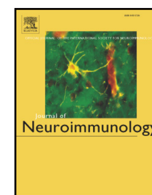

Suppression of microglia activation after hypoxia–ischemia results in age-dependent improvements in neurologic injury

Ulas Cikla [a,1], Vishal Chanana [a,1], Douglas B. Kintner [a], Lucia Covert [a], Taylor Dewall [a], Alex Waldman [a], Paul Rowley [a], Pelin Cengiz [a,b], Peter Ferrazzano [a,b,*]

[a] Waisman Center, University of Wisconsin, Madison, WI 53705, USA
[b] Department of Pediatrics, University of Wisconsin School of Medicine and Public Health, Madison, WI 53705, USA

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ABSTRACT

We previously found increased microglial proliferation and pro-inflammatory cytokine release in infant mice compared to juvenile mice after hypoxia–ischemia (HI). The aim of the current study was to assess for differences in the effect of microglial suppression on HI-induced brain injury in infant and juvenile mice. HI was induced in neonatal (P9) and juvenile (P30) mice and minocycline or vehicle was administered at 2 h and 24 h post-HI. P9 minocycline-treated mice demonstrated early but transient improvements in neurologic injury, while P30 minocycline-treated mice demonstrated sustained improvements in cerebral atrophy and Morris Water Maze performance at 60 days post-HI.

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1. Introduction

Neuroinflammation plays an important role in ischemic brain injury, and modulating the microglia-mediated inflammatory response to ischemia has been considered as a potential target for neuroprotective intervention. However, microglia exhibit a complex response to injury, releasing cytotoxic mediators which may worsen injury (Biran et al., 2006; Deng, 2010; Vexler and Yenari, 2009), and also expressing immunomodulatory and neurotrophic factors which contribute to healing and recovery from injury (Lalancette-Hebert et al., 2007). The exact contribution that each of these microglial responses plays to a specific injury likely varies depending on the mechanism, location, and severity of the injury, and importantly, may also depend on the age at which the injury occurs.

Microglia are derived from monocytes which originate in the yolk sac and populate the brain during early fetal brain development. In the fetal brain, these immature microglia are ameboid cells likely responsible for phagocytosis of cellular debris which results from brain development and synaptogenesis (Graeber and Streit, 2010). Perinatally, these microglia migrate throughout the brain and transition to a highly ramified immunosurveillance phenotype which is maintained throughout life. This process is thought to occur over the first 2–3 weeks of life in rodents and is complete by post-natal day 30. A growing body of literature has revealed differences in gene expression between neonatal and adult microglia (Butovsky et al., 2014; Parakalan et al., 2012). However, differences in how neonatal and mature microglia respond to injury remain poorly defined.

We previously described that microglia in the neonatal brain respond to hypoxia-ischemia (HI) with increased activation, proliferation, and release of pro-inflammatory mediators compared to juveniles (Ferrazzano et al., 2013). How the microglial response aggravates or ameliorates injury after hypoxia-ischemia in the developing brain has remained poorly understood. In order to understand age-dependent differences in microglial responses to ischemic brain injury, we determined the effect of microglial inhibition with minocycline on hypoxic-ischemic brain injury in neonatal (P9) and juvenile (P30) mice. Based on our previous findings of a more pro-inflammatory microglial response in neonatal brains, we hypothesized that suppression of microglial activation after HI would result in improved neuronal injury, less cerebral

Abbreviations: HI, Hypoxia ischemia; MAP2, microtubule associated protein 2; HBSS, Hank's balanced salt solution; PFA, paraformaldehyde; MWM, Morris Water Maze; IL, ipsilateral; CL, contralateral; MRI, Magnetic Resonance Imaging.

* Corresponding author at: Department of Pediatrics, University of Wisconsin Medical School, T517 Waisman Center, 1500 Highland Ave., Madison, WI, 53705.

E-mail addresses: cikla@wisc.edu (U. Cikla), chanana@pediatrics.wisc.edu (V. Chanana), dkintner@pediatrics.wisc.edu (D.B. Kintner), lcovert@wisc.edu (L. Covert), tdewall@wisc.edu (T. Dewall), awaldman@wisc.edu (A. Waldman), prawley@wisc.edu (P. Rowley), cengiz@pediatrics.wisc.edu (P. Cengiz), ferrazzano@pediatrics.wisc.edu (P. Ferrazzano).

[1] These authors contributed equally to this work.

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atrophy, and improved memory and learning in P9 mice compared to P30 mice.

2. Methods

2.1. Materials

Mouse monoclonal anti-microtubule associated protein 2 (MAP2) antibody was from Sigma (St. Louis, MO). Iba1 rabbit polyclonal antibody was from Wako Chemical (Richmond, VA). Rat anti-mouse CD45-FITC antibody was from AbD Serotec (Raleigh, NC). Mouse CD11b-APC conjugated antibody, goat anti-mouse Alexa Fluor 488-conjugated IgG and goat anti-rabbit Alexa Fluor 546-conjugated IgG were from Invitrogen (Carlsbad, CA). Vectashield mounting medium with DAPI was from Vector Labs (Burlingame, CA). Tissue-Tek O.C.T. compound was from Sakura Finetek (Torrance, CA). Hanks balanced salt solution (HBSS) was obtained from Mediatech Cellgro (Manassas, VA).

2.2. Animal usage

All procedures on animals were carried out in adherence with NIH Guide for the Care and use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Wisconsin-Madison.

2.3. Induction of hypoxia–ischemia

Using the Vannucci model of neonatal hypoxia–ischemia (Gurd et al., 1999), HI was induced in P9 and P30 mice as described previously (Ferrazzano et al., 2013). C57BL/6J mice (P9 and P30) were anesthetized with isoflurane (3% for induction, 1.5% for maintenance), in 30% O_2 and 70% N_2O. The duration of anesthesia was kept to less than 5 min in each animal to minimize any potential effects of isoflurane on neuronal injury or microglial activation (Wu et al., 2012; Xie et al., 2008; Zhang et al., 2012). The body temperature of the animal was maintained at 37 °C with a heated surgical platform (Patterson Scientific, Bend, OR). Under a Nikon SMZ-800 stereo surgical microscope (Nikon Instruments, Mellville, NY), a midline skin incision was made and the left common carotid artery was dissected out and electrically cauterized as described previously (Cengiz et al., 2011). The incision was superfused with 0.5% bupivacaine and closed with a 6.0 silk suture. Animals were returned to their dams and monitored continuously for the initial 30 min during a 2 h recovery period. The animals were then placed in a hypoxia chamber (BioSpherix Ltd., Redfield, NY) equilibrated with 10% O_2 and 90% N_2 at 37 °C for 50 min. In this model, the unilateral carotid artery cauterization by itself induces no injury because perfusion is maintained through collateral circulation. However, on subsequent exposure to hypoxia a unilateral ischemia occurs as blood flow decreases to the hemisphere ipsilateral to the cauterized carotid artery (Mujsce et al., 1990). After HI, animals were monitored continuously for the initial 30 min of reoxygenation and then checked every 30 min for 2 h and then daily until sacrificed. At 2 h and 24 h post-HI animals were given either minocycline (40 mg/kg, i.p. total volume = 0.2 ml) or vehicle (sterile saline).

2.4. Flow cytometry analysis of brain microglia cells

At day 2 or 9 after HI, mice were deeply anesthetized with 5% isoflurane plus N_2O and O_2 (3: 2) and decapitated. Immediately after decapitation, cerebellums and meninges were removed. The contralateral and ipsilateral hemispheres were separated, and the hippocampus, cortex and striatum were dissected from each hemisphere and submerged into ice-cold Hank's balanced salt solution (HBSS). Samples of each brain region were pooled from 5 mice for analysis. Brain tissues were cut into small pieces and dissociated into a single cell suspension by gentle physical disruption and enzymatic digestion using a commercially available tissue dissociation kit according to manufacturer's instructions (Miltenyi Biotech Inc. Auburn, CA). Myelin was removed by centrifugation of the sample in 0.9 M sucrose in HBSS at 2200 g for 10 min at 4 °C and cells were rinsed in HBSS and collected after passing through a 40 μm membrane by centrifugation.

For flow cytometry analysis, the cell suspension was first incubated with 10% goat serum in 0.1 M PBS (pH 7.4) for 20 min at room temperature. After the incubation, cells were centrifuged at 200 g for 5 min. The supernatant was then aspirated and the pellet was suspended (~10^6 cells/ml) in 4% paraformaldehyde (PFA) in 0.1 M PBS and incubated for 30 min at room temperature on an orbital shaker. Cells were then centrifuged at 1000 *g* for 5 min and washed with 0.1 M PBS to remove excess fixation buffer. The fixed cells (~10^6 cells) in 0.1 ml PBS were incubated with APC-conjugated rat anti-mouse CD11b and FITC-conjugated rat anti-mouse CD45 antibodies (25 μl of 0.2 mg/ml) for 30 min in ice. Cells were rinsed with 0.1 M PBS by centrifugation for 5 min at 1000 g and pellets were suspended in 500 μl of 0.1 M PBS and acquired immediately with a FACSCalibur flow cytometer running CellQuest Pro software (BD Biosciences, San Jose, CA) with the following settings: Forward scatter V = E00, gain = 1.0, mode = Lin; Side scatter V = 399, gain = 1.25, mode = Lin; FL1 V = 572, gain = 1.0, mode = Log; FL4 V = 704, mode = Log. In each experiment, 10,000 events were acquired for analysis from each sample. $CD11b^+/CD45^+$ cells were quantified in each brain region and expressed as the ratio of ipsilateral to contralateral cell counts.

2.5. Immunofluorescence staining

Mice were deeply anesthetized with 5% isoflurane plus N_2O and O_2 (3:2) and transcardially perfused with 4% PFA in 0.1 M PBS. Brains were post-fixed in 4% PFA in 0.1 M PBS for 12 h, and subsequently cryoprotected with 30% sucrose in 0.1 M PBS for 24–36 h at 4 °C. The brains were frozen in an optimal cutting temperature compound for 10 min and cut into 35 μm coronal sections on a freezing microtome (Leica SM 2000R; Buffalo Grove, IL), and stored in antifreeze solution at −20 °C.

2.5.1. Neurological scoring

Sections at the level of 0.02 mm anterior from bregma (anterior), 1.70 mm posterior from bregma (hippocampal), and 2.70 mm posterior from the bregma (posterior) were selected from each brain and processed for neurological scoring. Sections were rinsed 3 × 10 min in 0.1 M Tris-buffered saline (TBS, pH 7.4), and incubated with a blocking solution (0.1% Triton X-100 and 3% goat serum in 0.1 M TBS) for 30 min at 37 °C. Sections were incubated with mouse anti-MAP2 (1:500) in blocking solution for 1 h at 37 °C and then overnight at 4 °C. After rinsing 3 × 10 min in 0.1 M TBS, sections were incubated with goat anti-mouse Alexa Fluor 488-conjugated IgG in blocking solution (1:200) for 1 h at 37 °C. Sections were rinsed 3 × 10 min in 0.1 M TBS and then mounted on slides with vectashield mounting media. For neurological scoring, stitched whole brain fluorescent images (FITC) were captured on a Zeiss upright epifluorescent photomicroscope equipped with a motorized stage, 2.5× lens and a CDD camera driven by Stereo Investigator software (MBF Bioscience, Williston, VT). A blinded investigator scored the whole brain images using a scoring system modified from Sheldon et al. (Sheldon et al., 1998). Briefly, ipsilateral and contralateral brain regions were compared (anterior cortex, dentate gyrus, CA1, CA2, CA3, hippocampal cortex, hippocampal striatum, posterior cortex) and each region assigned a score of 0–3 (0 = no loss of MAP2 staining, normal dendritic morphology; 1 = some loss of MAP2 staining, disturbed dendritic morphology, hippocampal shrinkage, ventriculomegaly and cortical thinning; 2 = moderate loss of MAP2 staining, disturbed dendritic morphology, hippocampal shrinkage, ventriculomegaly and cortical thinning; 3 = total loss of MAP2 staining, gross infarction and loss of structures within

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