Brain, Behavior, and Immunity xxx (2016) xxx–xxx

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Glial cell morphological and density changes through the lifespan of rhesus macaques

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ARTICLE INFO

Article history:
Received 22 September 2015
Received in revised form 4 January 2016
Accepted 12 January 2016
Available online xxxx

Keywords:
Aging
Glia
Astrocyte
Microglia

ABSTRACT

How aging impacts the central nervous system (CNS) is an area of intense interest. Glial morphology is known to affect neuronal and immune function as well as metabolic and homeostatic balance. Activation of glia, both astrocytes and microglia, occurs at several stages during development and aging. The present study analyzed changes in glial morphology and density through the entire lifespan of rhesus macaques, which are physiologically and anatomically similar to humans. We observed apparent increases in gray matter astrocytic process length and process complexity as rhesus macaques matured from juveniles through adulthood. These changes were not attributed to cell enlargement because they were not accompanied by proportional changes in soma or process volume. There was a decrease in white matter microglial process length as rhesus macaques aged. Aging was shown to have a significant effect on gray matter microglial density, with a significant increase in aged macaques compared with adults. Overall, we observed significant changes in glial morphology as macaques age indicative of astrocytic activation with subsequent increase in microglial density in aged macaques.

1. Introduction

Aging is a normal biological process with changes to numerous physiological functions (see recent review by (Didier et al., 2016) for similarities between primates and humans). Improvements in public health, social services, and healthcare systems worldwide are producing an older human population. Quality of life during aging is dependent upon complex interrelationships between psychosocial and physical health parameters. There are numerous stages in the human and nonhuman primate lifecycle, including infancy, adolescence, adulthood and old-age. Of these, adolescence is noted to be a window of opportunity for "re-wiring" of the nervous system, with reduced spine density on neurons (Spear, 2000). Both astrocytes and microglia are thought to have roles in influencing developmental synaptic pruning (Sofroniew and Vinters, 2010). The purpose of this study was to reveal specific changes in glia during the lifetime of uninfected primates. We chose the prefrontal cortex (PFC) as our region of interest due to its role in higher-order functioning, such as spatial recognition, working memory, and long-term memory. These are impacted during PFC remodeling in adolescence (Spear, 2000) and compromised in age-related neurodegenerative diseases (Czeh et al., 2008).

During normal aging, from juveniles, through adolescence, adulthood and into eugeric aging, the CNS maintains an anti-inflammatory environment, largely through the action of astrocytes (Renner et al., 2011; Yirmiya and Goshen, 2011) and microglia (Ramesh et al., 2013). In these normal conditions, both astrocytes and microglia have a highly ramified morphology, which, for microglia at least, is remarkably consistent across cortical layers (Kongsui et al., 2014). Both astrocytes and microglia assist in glutamatergic signaling, and so structural remodeling of glial processes impacts neuronal signaling (Mayhew et al., 2015). These non-inflammatory roles for glial cells may be part of the normal maturation process (Tremblay, 2011). Indeed, failure of synaptic pruning by microglia induces neurodevelopmental delay (Zhan et al., 2014), and microglial activation *in utero* can have life-long effects (reviewed by Edmonson et al., 2016).

Immune cells in the CNS have dual functionalities that can lead to damage of surrounding cells and tissues. Studies have frequently shown a connection between chronic inflammation or stress and

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http://dx.doi.org/10.1016/j.bbi.2016.01.006
0889-1591/

the onset of neurodegenerative diseases (Harry, 2013; Hinwood et al., 2013; Middeldorp and Hol, 2011; Orre et al., 2014; Prinz et al., 2011; Tynan et al., 2013). The increase in circulating proinflammatory cytokines in aging humans and nonhuman primates also adversely impacts the CNS. Neural structure and volume are associated with circulating pro-inflammatory cytokines, including IL-6 (Willette et al., 2010), IL-8 and IL-10 (Willette et al., 2013), and combined with increased oxidative stress (Ungvari et al., 2011), indicate a link between chronic inflammation and activation in the CNS.

Microglia are considered the resident macrophages of the brain (Ramesh et al., 2013). These highly ramified cells continuously survey the CNS for abnormalities and may become phagocytic or extend their processes to isolate damaged sites. During infection or injury, microglia can become activated into either a classical (pro-inflammatory) M1 phenotype or alternative (anti-inflamm atory) M2 phenotype, depending on signals received from astrocytes and T-cells. Primed microglia can also produce an exaggerated inflammatory response to a subsequent stimulus (Perry and Holmes, 2014; Renner et al., 2012). The inability of microglia to successfully clear cell debris can induce prolonged inflammation and ultimately lead to depression (Yirmiya et al., 2015), neurodegeneration and premature aging (Harry, 2013; Schwartz et al., 2013).

Because of the close physiological and genetic relationship with humans, studies using nonhuman primates (NHPs) have been invaluable for understanding the changes in brain structure and function that take years, rather than months, to develop (reviewed recently by (Didier et al., 2016)). Thus, the nonhuman primate brain may be an ideal model for eugeric, or normal aging, especially in brain. Rhesus monkeys age approximately 4 times faster than humans (Colman and Anderson, 2011; Kohama et al., 2012; Spear, 2000), providing a unique opportunity to examine glial changes in primates as they age. Importantly, primates are unique in having a distinct adolescent phase of development after puberty, but before true adulthood (Goldman-Rakic, 1987; Spear, 2000; Verrico et al., 2011).

While previous studies have focused on glial activation within one age group or between diseased and controlled states (Lee et al., 2014, 2013a,b; Snook et al., 2013), the present study attempts to identify changes incurred during the entire lifespan in otherwise healthy macaques. Our data will show a sharp increase in the branching, and hence connectivity of glia during the transition from adolescence to adulthood. This occurred concurrently with decreased microglial arbor length. Finally, we noted increased numbers of IBA1 immunopostive cells that were morphologically consistent with microglia in geriatric macaques.

2. Materials and methods

2.1. Ethics statement, animal housing, & selection of tissues

Those animals housed indoors were maintained in Animal Biosafety Level 2 housing with a 12:12-h light:dark cycle, relative humidity 30–70%, and a temperature of 17.8–28.9 °C. Water was available *ad libitum*, and a standard commercially formulated nonhuman primate diet (Lab Fiber Plus Monkey DT, 5K63, PMI Nutrition International, St. Louis, MO) was provided twice daily and supplemented daily with fresh fruit and/or forage material as part of the environmental enrichment program. All animals at TNPRC have environmental enrichment, widely used to improve welfare in captive macaques. Each cage (Allentown, Inc., Allentown, NJ) measured 36 inches (91.4 cm) in height with 8.6 square feet (0.8 square meters) of floor space and contained a perch, a portable enrichment toy, a mirror, and a forage board for feeding enrichment. Practices in the housing and care of animals conformed to the regulations and standards of the U.S. Department of Health and Human Services Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals, and the Guide for the Care and Use of Laboratory Animals. The Tulane National Primate Research Center (TNPRC; Animal Welfare Assurance #A4499-01) is fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care-International. All animals are routinely cared for according to the guidelines prescribed by the NIH Guide to Laboratory Animal Care. The TNPRC conducts all research in accordance with the recommendations of the Weatherall report – "The use of non-human primates in research". The Institutional Animal Care and Use Committee (IACUC) of the Tulane National Primate Research Center approved all animal-related protocols, including any treatments used with nonhuman primates. All animal procedures were overseen by veterinarians and their staff.

Animals were humanely euthanized by the veterinary staff at the TNPRC in accordance with endpoint policies. Euthanasia was conducted by anesthesia with ketamine hydrochloride (10 mg/kg) followed by an overdose with sodium pentobarbital and immediate necropsy. This method was consistent with the recommendation of the American Veterinary Medical Association guidelines (Lee et al., 2013b). Three brain regions approximately 1 cm thick are routinely collected during necropsy of colony animals at TNPRC representing frontal lobe, parietal & temporal lobe /thalamus/basal ganglia, and cerebellum/occipital lobe. All tissues are fixed at routine necropsy by immersion in 10% neutral buffered formalin with zinc modification for 48 h before trimming and paraffin embedding.

The present study stained, imaged, and analyzed formalin fixed paraffin embedded frontal lobe sections collected in the past eight years and stored at the TNPRC tissue archive. All 19 animals constituting this study were uninfected Rhesus macaques that went to necropsy as a result of investigator-initiated control animals, trauma or natural causes. These included 8 males and 11 females ages 0.45–25.59 years old (Table 1). Our animals were divided into four groups: juveniles (5 months to 2 years), adolescents (approximately three to five years), adults (seven to twelve years) and geriatrics (twenty years or older) based on general developmental stages (Colman and Anderson, 2011; Kohama et al., 2012; Spear, 2000).

2.2. Immunohistochemistry

Formalin fixed paraffin embedded frontal lobe tissue samples were cut 6 μm thick using an Ultramicrotome. These tissue samples were deparaffinized with Xylene for 25 min and rehydrated with decreasing concentrations of ethanol and deionized water. Samples were then incubated in two solutions of Sodium Citrate Buffer: the first solution at room temperature for 30 min and the second at >95° for 30 min. Tissue samples were allowed to cool in a shaker for 20 min before outlined in wax and incubated with DAKO protein block at room temperature for one hour. Next, the protein block was removed, and each sample was incubated overnight at 4 °C with 150 μL of a 1:250 dilution of Ionized Calcium-Binding Adaptor Molecule 1 (Iba-1) primary antibody solution (Wako, Richmond, VA). The next day, samples were washed three times in Tris-Buffered Saline (TBS), and each was covered in 150 μL of 1:1000 Alexa 488 Anti-Rabbit secondary antibody. After one hour, the slides were washed three times with TBS and incubated with DAKO block for one hour. After removing the protein block, each sample was incubated for one hour with 150 μL of 1:250 cy-3 pre-conjugated Glial Fibrillary Acidic Protein (GFAP) antibody (clone GA-1, Sigma). Following this, the samples were washed a final three times in TBS. All samples were coverslipped using Prolong Gold with DAPI and glass slide covers. The slides were refrigerated until imaged by fluorescent microscopy.

Please cite this article in press as: Robillard, K.N., et al. Glial cell morphological and density changes through the lifespan of rhesus macaques. Brain Behav. Immun. (2016), http://dx.doi.org/10.1016/j.bbi.2016.01.006