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DISTINCT ACTIVATION PROFILES IN MICROGLIA OF DIFFERENT AGES: A SYSTEMATIC STUDY IN ISOLATED EMBRYONIC TO AGED MICROGLIAL CULTURES

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Abstract—Microglia have been implicated in disease progression for several age-related brain disorders. However, while microglia's contribution to the progression of these disorders is accepted, the effect of aging on their endogenous cellular characteristics has received limited attention. In fact, a comprehensive study of how the structure and function of microglia changes as a function of developmental age has yet to be performed. Here, we describe the functional response characteristics of primary microglial cultures prepared from embryonic, neonatal (Neo), 2–3 month-old, 6–8 month-old, 9–11 month-old, and 13–15 month-old rats. Microglial morphology, glutamate (GLU) uptake, and release of trophic and inflammatory factors were assessed under basal conditions and in microglia activated with adenosine 5′-triphosphate (ATP) or lipopolysaccharide. We found that microglia from different age groups were both morphologically and functionally distinct. Upon activation by ATP, Neo microglia were the most reactive, upregulating nitric oxide, tumor necrosis factor-α, and brain-derived neurotrophic factor release as well as GLU uptake. This upregulation translated into neurotoxicity in microglia-neuron co-cultures that were not observed with microglia of different developmental ages. Interestingly, 13–15 month-old microglia exhibited similar activation profiles to Neo microglia, whereas microglia from younger adults and embryos were activated less by ATP. Our data also identify age-dependent differences in purinergic receptor subtype expression that contribute to the regulation of neuronal survival. Combined, our data demonstrate that microglial activation and purinergic receptor profiles vary non-linearly with developmental age, a potentially important finding for studies examining the role of microglia in neurodegenerative disorders. © 2013 Published by Elsevier Ltd. on behalf of IBRO.

Key words: development, ageing, purinergic receptors, inflammation, cytokines, neurotoxicity.

INTRODUCTION

Microglia are the resident immune cells of the brain. Developmentally derived from the bone marrow, they exist in a 'ramified' state in the healthy central nervous system (CNS). During injuries or other CNS disturbances, microglia respond to signals from the site of injury or disease and become 'activated'. The response of activated microglia to CNS injuries typically consists of proliferation, migration to the injury site, secretion of immune mediators, and phagocytizing of toxic substances and cellular debris (Raivich, 2005; Streit et al., 2005; Lai and Todd, 2006; Lai et al., 2009). Activated microglia are capable of producing both toxic and trophic effectors and may thereby promote neurotoxicity or neuroprotection, respectively, depending on the nature of the activating signal (Nakajima and Kohsaka, 2004; Hanisch and Kettenmann, 2007). Additionally, there are intrinsic differences among microglial populations that are independent of the activating signal. Microglia's region of origin, gender, and age have been shown to affect their phenotype (Lawson et al., 1990; Ren et al., 1999; Yu et al., 2002; Floden et al., 2005; Carson et al., 2007; Sierra et al., 2007; de Haas et al., 2008; Wu et al., 2008; Crain et al., 2009; Lai et al., 2011; Njie et al., 2012). The effects of age are particularly intriguing due to the contribution of microglia to the progression of age-related disorders such as Alzheimer's disease. Microglia express receptors for numerous signaling molecules and are extremely sensitive to changes in the neurochemical environment of the surrounding brain tissue (Hanisch and Kettenmann, 2007). As brain chemistry changes with age, microglia may respond to associated changes in their neurochemical environment by transforming their phenotype. Supporting this, previous studies have cultured microglia derived from neonatal (Neo), young, and aged animals and found differences in their responses to activation (Floden et al., 2005; Njie et al., 2012). However, nearly all of these studies made their comparisons between two age groups separated by a

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Abbreviations: AG, aminoguanidine; ANOVA, analysis of variance; ATP, adenosine 5′-triphosphate; BBG, Brilliant Blue G; BDNF, brain-derived neurotrophic factor; CM, conditioned media; DMEM, Dulbecco's Modified Eagle Medium; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assays; Em, embryonic; FBS, fetal bovine serum; GFAP, glial fibrillary acidic protein; GLU, glutamate; Hyp, hypoxia; Iba1, ionized calcium-binding adaptor protein 1; iNOS, inducible NO synthase; LPS, lipopolysaccharide; MAP-2, microtubule-associated protein-2; Neo, neonatal; NO, nitric oxide; PBS, phosphate-buffered saline; RB2, Reactive Blue 2; TNF-α, tumor necrosis factor-α.

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large age gap, and typically excluded microglia derived from middle-aged adults and developing embryos. It is thus difficult to construct a complete picture of the effects of developmental age on microglial activation profiles. Considering the widespread use of Neo microglial cultures for research, and the inferences on brain function and degeneration derived from those studies, a better understanding of phenotypic variations across developmental ages is essential for optimal translation of culture findings. Here, we examined the activation response profiles of microglia derived from six different age groups of Sprague–Dawley rats: embryonic (Em) day 18, Neo 1-day-old, 2–3-month (mo)-old, 6–8-mo-old, 9–11-mo-old, and 13–15-mo-old. Microglia from all age groups were stimulated with the activating agents adenosine 5′-triphosphate (ATP) and lipopolysaccharide (LPS). Microglial morphologies, effector profiles after activation, and consequences for neuronal survival were assessed. In addition, purinergic receptor expression and neurochemical conditioning were examined as possible mechanisms that underlie the development of age-specific activation profiles.

EXPERIMENTAL PROCEDURES

Primary neuronal and microglial cultures

Neuronal cultures were prepared from cortices of Em day 18 Sprague–Dawley rats using methods derived from previous studies (Brewer et al., 1993; Swayze et al., 2004). In brief, dissected cortices were dissociated by 0.25% (trypsin–EDTA, Gibco) digestion and mechanical titrations. Dissociated neurons were suspended in B-27 supplemented Neurobasal Media (Gibco) and plated at a density of 2×10^5 cells per well on poly-L-lysine (Sigma)-pre-coated 24-well plates. Contaminating glial cells were minimized by treatment with 1 μM cytosine arabinoside (Sigma) from days *in vitro* (DIV) 3 to DIV 6. This yielded neuronal-enriched cultures that were verified to be >98% neurons and <2% astrocytes using immunohistochemistry for cell-type-specific markers (the neuronal marker microtubule-associated protein-2 (MAP-2), the astrocyte marker glial fibrillary acidic protein (GFAP), the microglial marker ionized calcium-binding adaptor protein 1 (Iba1), and the oligodendrocyte marker CNPase). Experiments were performed on cultures that were 10–13 DIV.

Embryonic and Neo microglial cultures were first isolated as mixed cultures prepared from the brains of Sprague–Dawley rats. The dissected brains were dissociated by enzymatic digestion with 0.25% trypsin–EDTA and mechanical titrations. Dissociated cells were suspended in Dulbecco's Modified Eagle Medium (DMEM)/F12 supplemented with 10% fetal bovine serum (FBS, Hyclone), then plated on poly-L-lysine-coated 6-well or 24-well plates at a density of 4×10^6 cells and 1×10^6 per well respectively. After 18–22 DIV, microglial cells were isolated from these mixed cultures using a previously reported high-yield method (Saura et al., 2003), which uses a 1:1 mixture of 0.25% trypsin–EDTA: DMEM to dissociate non-microglial cells from the surface. The remaining adherent cells were verified to be >95% microglia by cell type-specific markers. The isolated microglial cultures were kept in serum-free DMEM for an additional 1–2 DIV before experiments were performed.

To culture adult microglia, a Percoll (Sigma)-based method (Cardona et al., 2006) was used. In brief, brains of adult Sprague–Dawley rats of various ages were isolated and mechanically chopped to small pieces of approximately 1-mm length while on ice. The chopped pieces were then enzymatically digested by 0.25% trypsin–EDTA (Gibco) and gentle mechanical titurations with minimal pressure in DMEM/F12 with 10% FBS. The cell suspension was centrifuged to a pellet and resuspended in 37% Percoll (diluted in DMEM/F12). This Percoll fraction with cells was then carefully laid on top of a 70% Percoll layer. Afterward, a 30% layer followed by a 0% (DMEM/F12 only) layer was sequentially laid on top of the 37% layer. The tube with a 70%-37%-30%-0% gradient was centrifuged at 500*g* for 45 min. After centrifugation, the interface between the 70% and the 37% layer, which contained microglia, was collected using a Pasteur pipet. The collected portion was diluted five times in DMEM/F12, mixed well, and then centrifuged at 3000*g* to remove leftover Percoll. The remaining pellet was suspended in DMEM/F12 with 10% FBS and plated at a density of 1/4 plates per brain. This yields a microglial density comparative to that in Em and Neo microglial cultures. After one DIV, the cultures were switched to a serum-free DMEM/F12, and were used for experiments after another DIV. The cultures were verified to be >95% microglia using cell-type-specific markers.

Chemicals

Unless otherwise mentioned, all chemicals were purchased from Sigma. Concentrations used for aminoguanidine (AG, 300 μM), Brilliant Blue G (BBG, 1 μM), Reactive Blue 2 (RB2, 30 μM), ATP (0.01–1 mM), and LPS (1 μg/mL) were based on previous *in vitro* studies (Lockhart et al., 1998; Nakajima et al., 2001; Inoue, 2002; Suzuki et al., 2004; Koizumi et al., 2007).

Immunocytochemistry and morphological analysis

Mouse anti-MAP-2 (1:500) and mouse anti-GFAP (1:1000) were purchased from Sigma. Mouse anti-CNPase (1:500) and rabbit anti-Iba1 (1:1000) were purchased from Chemicon and Wako, respectively. Fluorescence-conjugated secondary antibodies (1:500) were obtained from Jackson ImmunoResearch. Immunocytochemistry was performed using previously described methods (Lai and Todd, 2008; Lai et al., 2011). Cultures were first fixed in 2% paraformaldehyde for 10 minutes (min). After 1 hour (h) blocking and permeablization with 10% horse serum and 0.25% Triton, they were labeled with primary antibodies for 1 h, then with the appropriate fluorescence-conjugated secondary antibodies for 30 min. Morphological analysis using ImageJ (NIH) was performed on images of Iba-1-labeled microglia acquired using epifluorescent

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