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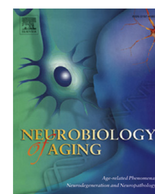

Age and duration of inflammatory environment differentially affect the neuroimmune response and catecholaminergic neurons in the midbrain and brainstem

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ABSTRACT

Neuroinflammation and degeneration of ascending catecholaminergic systems occur early in the neurodegenerative process. Age and the duration of a pro-inflammatory environment induced by continuous intraventricular lipopolysaccharide (LPS) differentially affect the expression profile of pro- and anti-inflammatory genes and proteins as well as the number of activated microglia (express major histocompatibility complex II; MHC II) and the integrity and density of ascending catecholaminergic neural systems originating from the locus coeruleus (LC) and substantia nigra pars compacta (SNpc) in rats. LPS infusion increased gene expression and/or protein levels for both pro- and anti-inflammatory biomarkers. Although LPS infusion stimulated a robust increase in IL-1ß gene and protein expression, this increase was blunted with age. LPS infusion also increased the density of activated microglia cells throughout the midbrain and brainstem. Corresponding to the development of a pro-inflammatory environment, LC and SNpc neurons immunopositive for tyrosine-hydroxylase (the rate-limiting synthetic enzyme for dopamine and norepinephrine) decreased in number, along with a decrease in tyrosine-hydroxylase gene expression in the midbrain and/or brainstem region. Our data support the concept that continuous exposure to a pro-inflammatory environment drives exaggerated changes in the production and release of inflammatory mediators that interact with age to impair functional capacity of the SNpc and LC.

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1. Introduction

Activation of the brain's resident microglia occurs during normal aging, is associated with many neurodegenerative diseases such as Parkinson's disease (PD) and Alzheimer's disease (AD), and may drive a self-propagating toxic cycle promoted by the release of pro-inflammatory and loss of protective mediators (Aarsland et al., 2001; Akiyama et al., 2000; Bartels and Leenders, 2005; Block and Hong, 2005; Cribbs et al., 2012; Griffin et al., 1989; Hobson and Meara, 2004; Hughes et al., 2000; Swardfager et al., 2010; Whitton, 2007). When these processes are triggered within vulnerable brain regions, they may lead to the loss of acetylcholinergic neurons in the nucleus basalis magnocellularis (Whitton, 2007; Willard et al., 1999) as well as dopaminergic neurons in the substantia nigra pars compacta (SNpc), noradrenergic (NE) neurons in the locus coeruleus (LC) and, all regions that show significant early cell loss in the brains of patients with PD and AD (Braak et al., 2003; Grudzien et al., 2007; Halliday et al., 2006; Rudow et al., 2008; Szot et al., 2006).

We and others have speculated that the consequences of neuroinflammation associated with microglial activation, are carefully regulated until, because of normal aging or the deposition of toxic proteins, there is a gradual shift to a nonequilibrium state that is permissive for neurodegenerative processes (Block and Hong, 2005; Colton and Wilcock, 2010; Smith et al., 2012; Wenk and Hauss-Wegrzyniak, 2001). Microglia can assume various phenotypes that are associated either with the release of potentially destructive, pro-inflammatory cytokines, and other toxic molecules or the expression of a cytokine profile that sustains repair, recovery, and growth. Microglia in various states of activation are detectable many years before the onset of neuropathological changes (Cagnin et al., 2006; Gerhard et al., 2006; Imamura et al., 2003). Because vulnerable brain regions are likely exposed for many decades to a complex combination of microglia in various activation states (Bilbo, 2010; Eikelenboom et al., 2010; Heneka et al., 2010; Herrup, 2010). The present study investigated the differential influence of

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brain age and the duration of the pro-inflammatory environment upon the expression of pro- and anti-inflammatory genes and proteins as well as the number of activated microglia and the integrity and density of ascending catecholaminergic neural systems originating in the LC and SNpc.

2. Methods

2.1. Experimental design

Young (3 months), middle-aged (9 months), and aged (23 months) male F-344 rats (Harlan Sprague–Dawley) received chronic infusion of lipopolysaccharide (LPS) or its vehicle (artificial cerebral spinal fluid; aCSF) into the fourth ventricle for 21 or 56 days. We believe that this approach best represents the situation present during the early stages of many chronic neurodegenerative diseases. Multiple counter-balanced iterations of this study were performed to produce a total of 132 rats; yielding experimental groups with a minimum of 11 rats that were divided between biochemical (minimum 6 rats/group) and histologic (minimum 5 rats/group) analysis. Midbrain and/or brainstem regions were evaluated for protein and messenger RNA (mRNA) expression of inflammatory markers and the LPS receptor (toll-like receptor 4; TLR4), as well as the presence of MHC II-IR microglia, which was used to define activated microglia. Changes in these immune factors at 3 ages and after short (21 days) or long (56 days) of continuous LPS infusion were then evaluated with respect to changes in neurotransmitter systems including expression of genes involved in the regulation of glutamate (glutamate transporter 1; GLT1, and the cystine-glutamate anti-porter; XcT), and gene and histologic expression of the enzymes responsible for production of dopamine (tyrosine hydroxylase; TH) and norepinephrine (dopamine-ß-hydroxylase; DBH). DBH immunostaining was examined to determine specifically the integrity of norepinephrine innervation of the dentate gyrus region of the hippocampus, which does not receive a dense dopaminergic input (Gasbarri et al., 1994). TH immunostaining was used to define specifically the norepinephrine neurons in the LC and dopaminergic neurons in the SNpc.

2.2. Subjects

Rats were maintained on a 12/12-hours light–dark cycle with lights off at 09:00 in a temperature-controlled room (22 °C) with free access to food and water. All rats were sacrificed during the dark phase of the diurnal cycle. Body weights and general health were closely monitored throughout the study. All rats were allowed at least 1 week to adapt to their new environment before surgery. The experiments were carried out in accordance with the National Institute of Health Guide for the Care and Use of Laboratory Animals (NIH Publications No. 80-23) revised 1996, and formal approval to conduct the experiments was obtained from the animal subjects review board from The Ohio State University.

2.3. Surgery

aCSF (140 mM NaCl, 3.0 mM KCl, 2.5 mM $CaCl_2$, 1.0 mM $MgCl_2$, and 1.2 mM Na_2HPO_4 adjusted to pH 7.4) or LPS (0.25 μg/h, 1.66 mg/mL prepared in aCSF; Escherichia coli, serotype 055:B5, TCA extraction, Sigma-Aldrich, St. Louis, MO, USA) were continuously infused via a cannula implanted into the fourth ventricle (−2.5 mm anterior-posterior and −7.0 mm dorsal-ventral, relative to lambda) and attached (via Tygon tubing, 0.06 O.D.) to an osmotic minipump (Alzet model #2006, to deliver 0.15 μl/h; DURECT Corporation, Cupertino, CA, USA) as previously described (Hauss-Wegrzyniak et al., 1998; Marchalant et al., 2007; Rosi et al., 2004). The average fill volume and release rates for the pump allows for an infusion up to 56 days. Postoperative care included lidocaine 1% solution applied to the exposed skin upon closure, 2 mL of isotonic saline by subcutaneous injection to prevent dehydration during recovery and 2% tylenol in the drinking water for 3 days before and after surgery.

2.4. Tissue collection

Rats used for protein and mRNA analysis were briefly anesthetized and then rapidly decapitated; their midbrain and/or brainstem (extending from just rostral to the SNpc and just caudal to the LC) and entire hippocampus were quickly dissected on ice and stored at −80 °C until processed. Blood was collected during the rapid decapitation procedure. After centrifugation at 4 °C for 15 minutes at 2500 ×g, serum was collected and assayed. Rats used for immunohistochemistry were deeply anesthetized with isoflurane for a transcardiac perfusion with 80 mL of cold 0.9% saline containing 50 U/mL heparin, followed by 120 mL of 4% paraformaldehyde in 0.1 M phosphate buffered saline (PBS), pH 7.4, at a rate of 10 mL/min. Brains were postfixed overnight in the same fixative and then stored in PBS.

2.5. Protein analysis

Brainstem or serum levels of tumor necrosis factor (TNF)-α, interleukin-1 (IL-1)-α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12 IL-13, IL-18 (interferon-γ inducing factor), interferon-γ, and granulocyte-macrophage-stimulating factor (GM-CSF) were quantified simultaneously with a magnetic bead-based immunoassay (Bio-Rad, BioPlex Pro Rat Standard, 171-K1002M), according to the manufacturer's protocol. Briefly, total protein was extracted from frozen midbrain and/or brainstem with a BioPlex Cell Lysis Kit (BioRad, Richmond, CA, USA). A mixture of distinct capture beads (fluorescently dyed microspheres) each with a specific spectral address and conjugated to an antibody against one of the cytokines listed previously were dispensed across a 96-well plate and protected from light. Samples and antigen standards were added in duplicate and the plate was shaken (700 RPM, 1 hour). Then a mixture of biotinylated detection antibodies directed against each of the primary antibodies was added and the plate was shaken (700 RPM, 30 minutes); unbound materials were washed away (3 times) (BioRad, BioPlex Pro wash station). Each well was then incubated with a streptavidin-phycoerythrin conjugate reporter dye that binds to the detection antibody (700 RPM, 10 minutes); unbound materials were washed away (3 times). Each well was then suspended in assay buffer and shaken (1100 RPM for 30 seconds). Finally, the contents were passed through a dual detection multiplexing machine (Bio-Rad MAGPIX multiplex reader) with a classification laser that distinguishes each of the proteins by color of its bound antigen-specific bead and a reporter laser that quantifies each molecule based upon the fluorescence of bound antigen-specific streptavidin-phycoerythrin conjugate reporter dye. Values were standardized to protein content of the homogenate obtained with a Bio-Rad protein assay (Bio-Rad), and results are reported as pg/mg protein.

2.6. Real-time polymerase chain reaction mRNA analysis

Brainstems were evaluated for mRNA expression of: TNFα, IL1ß, transforming growth factor-β (TGFß), TLR4, fractalkine receptor (CX3CR1), GLT1, XcT, brain-derived neurotrophic factor (BDNF). TH was evaluated in the midbrain and/or brainstem as a precursor to LC norepinephrine as well as SNpc dopamine, and DBH was evaluated in the hippocampus because it is a precursor to norepinephrine that can distinguish input from the LC projections. Tissues were homogenized in Trizol (Life Technologies, Carlsbad, CA, USA). Total RNA

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