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Differential effects of homotypic vs. heterotypic chronic stress regimens on microglial activation in the prefrontal cortex

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HIGHLIGHTS

- Chronic restraint stress produces microglial activation in the prefrontal cortex.
- Chronic variable stress does not produce microglial activation.
- Chronic restraint stress, but not variable stress, produces HPA axis habituation.
- Microglial activation may be an adaptive process.

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ABSTRACT

Stress pathology is associated with hypothalamic–pituitary–adrenal (HPA) axis dysregulation and aberrant glucocorticoid responses. Recent studies indicate increases in prefrontal cortical ionized calcium-binding adapter molecule 1 (Iba-1) staining following repeated restraint, reflecting increased microglial densities. Our experiments tested expression of Iba-1 staining in the prelimbic cortex (PL), infralimbic cortex (IL) and the hypothalamic paraventricular nucleus (PVN) following two-week exposure to repeated restraint (RR) and chronic variable stress (CVS), representing homotypic and heterotypic regimens, respectively. Unstressed animals served as controls. We specifically examined Iba-1 immunofluorescence in layers 2 and 3 versus layers 5 and 6 of the PL and IL, using both cell number and field staining density. Iba-1 field staining density was increased in both the PL and IL following RR in comparison to controls. This effect was not observed following CVS. Furthermore, PVN Iba-1 immunoreactivity was not affected by either stress regimen. Cell number did not vary within any brain areas or across stress exposures. Changes in microglial field density did not reflect changes in vascular density. Increases in PL and IL microglial density indicate selective microglial activation during RR, perhaps due to mild stress in the context of limited elevations in anti-inflammatory glucocorticoid actions. This research was supported by NIH grants [MH049698 and MH069860].

1. Introduction

Chronic stress plays a prominent role in triggering or enhancing many psychiatric disorders, including major depressive disorder and schizophrenia [1–3]. Stress-responsive brain regions and limbic circuitry are receiving wide attention as potential mediators of chronic stress pathology. The medial prefrontal cortex (mPFC) is highly stress reactive and undergoes significant changes in morphology and function following chronic stress [3,4]. These changes align with behavioral phenotypes in rats that are consistent with depression symptoms in humans [4,5,7].

Many of the symptoms associated with depression are accompanied by immune activation. Depressed mood, anhedonia, weight change, and fatigue are characteristics of depression, but are also related to sickness behavior [6]. Chronic restraint stress induces microglial hypertrophy in the PFC, hippocampus, and nucleus accumbens [7], suggestive of inflammatory reactions in the brain. Morphological changes in PFC microglia following chronic restraint stress are correlated with increases in neuronal ΔFosB and deficits in the T-maze; these effects are ameliorated by the microglial inhibitor minocycline [5].

Microglia have a dynamic morphology that is indicative of their function in response to various stimuli. Most microglia in the healthy brain are in a resting or ramified state, characterized by small cell bodies and thin processes [8]. However, ramified microglia are constantly surveying the environment and undergo rapid morphological changes to exert a variety of functions [8]. Microglia react to signals of

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neuronal damage and maintain tissue homeostasis. Resting microglia transition to a hyper-ramified state after mild stimulation, characterized by a thickening and branching of secondary processes. Physical tissue injury induces microglia to withdraw their processes to become reactive and then phagocytic [9]. Chronic stress stimulates microglia into hyper-ramification, which may represent a novel response to stress and unique microglial phenotype with mechanisms not yet fully understood. Hyper-ramification of microglia during stress may be associated with local inflammation, which can contribute to neurodegeneration, demyelination, and synaptic dysfunction [10]. For example, microglia have been implicated in actively pruning hippocampal synapses in the developing brain [11,12].

The glucocorticoid stress response is initiated by the hypothalamic–pituitary adrenocortical (HPA) axis. The paraventricular nucleus (PVN) of the hypothalamus initiates a hormonal cascade that stimulates adrenal synthesis and release of glucocorticoids (GCs). Peripherally, GCs are potently immunosuppressant. Centrally, GCs can either activate or inhibit microglial activity in various contexts, ranging from in vitro, in vivo, acute, chronic, in combination with LPS, etc. [13–15]. The effects of chronic stress-induced microglial activation are not well understood, despite the GC receptor (GR) being among the most abundant microglial steroid receptors [13]. To identify the role of GCs and microglia in chronic stress, the current study examined microglial responses to two separate stress regimens that induce distinct GC profiles. Tynan et al. demonstrated microglial hyper-ramification following repeat restraint stress, while measuring sucrose preference, body weight, and body temperature but not corticosterone [7]. Repeated restraint stress causes significant HPA axis habituation over the course of several exposures [16], and basal corticosterone secretion, adrenal hypertrophy, and thymic atrophy are generally less than those observed in non-habituating regimens [17]. These data raise questions regarding the possible role of microglia in adaptive vs. pathological consequences of chronic stress. The current study provides a side-by-side test of the impact of habituating vs. non-habituating stress protocols as a means of determining what type of stressor exposure causes microglial activation.

2. Materials and methods

2.1. Subjects

Male Sprague-Dawley rats from Harlan (Indianapolis, IN, USA), weighing 250–275 g on arrival, were housed two per cage in clear polycarbonate cages with granulated corncob bedding. Food and water were available ad libitum. The colony room was temperature and humidity controlled with a 12-hour light cycle (lights on 6:00 am; lights off 6:00 pm). Rats acclimated to the colony facility for 1 week prior to experimental manipulations. Subjects were randomly assigned to one of three groups: control unhandled (CON, n = 10), repeat restraint stress (RR, n = 10), and chronic variable stress (CVS, n = 12). All experimental procedures were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Animals and approved by the University of Cincinnati Institutional Animal Care and Use Committee.

2.2. Stress regimens

For RR, subjects were fixed into Plexiglas restraint tubes for 30 min every morning at 10:00 for 14 consecutive days. The CVS regimen lasted 14 consecutive days, with two stressors every day, and was run concurrently with the RR group. Morning stressors were applied any time between 08:00 and 11:30 h, while afternoon stressors were applied any time between 13:30 and 17:00 h. They included: hypoxia (30 min in 8% O_2/92% N_2), rotation stress (1 h at 100 rpm on a platform orbital shaker), warm swim (20 min at 31 °C), cold swim (10 min at 18 °C), cold room (1 h at 4 °C), and overnight crowding. On the morning of the 15th day, the rats were administered an overdose of sodium pentobarbital (150 mg/kg) and perfused with phosphate-buffered saline, followed by 4% paraformaldehyde. Brains were postfixed overnight in 4% paraformaldehyde and transferred to 30% sucrose (4 °C).

2.3. Immunohistochemistry

Using a sliding microtome, 30 μm sections were cut and stored at −20 °C in cryoprotectant (0.1 M phosphate buffer, 30% sucrose, 1% polyvinylpyrrolidone, and 30% ethylene glycol). Sections were transferred from cryoprotectant into 50 mM potassium phosphate-buffered saline (KPBS) (pH 7.2) at room temperature (RT). After rinsing off the cryoprotectant with KPBS (5 × 5 min), sections were incubated for 1 h at RT in blocking solution (50 mM KPBS, 0.1% bovine serum albumin, 0.2% Triton X-100). Immediately thereafter, sections were incubated for 16 h at RT in primary anti-Iba-1 polyclonal rabbit antibody for visualizing microglia (1:1500; Synaptic Systems; Goettingen, Germany) and primary anti-NeuN monoclonal mouse antibody (1:200; Millipore, Temecula, CA, USA) for identifying the boundaries of the PVN and layers of the PFC. Dilutions were in blocking solution (50 mm KPBS, 0.1% bovine serum albumin, and 0.2% Triton X-100). Sections were rinsed (5 × 5 min) in KPBS and then incubated for 1 h at RT in both Cy 3 donkey anti-rabbit 1:500 (Jackson Immuno Research, West Grove, PA, USA) and Alexa 488 goat anti-mouse 1:500 (Molecular Probes, Eugene, OR, USA), for microglia. For blood vessels, the Alexa 488 secondary was used in conjunction with DyLight 594 labeled *Lycopersicon esculentum* (Tomato) Lectin (1:400; Vector, Burlingame, CA, USA). Dilutions were with blocking solution. Sections were rinsed (4 × 5 min) with KPBS and mounted onto slides. Dried slides were rinsed with Nano-H_20 and coverslipped with Fluka mounting medium (Sigma Aldrich, St. Louis, MO, USA) to be used for imaging.

2.4. Imaging

The Zeiss Axiovision 4.6 software was used for all image quantification and anatomical landmarks were determined using features described in the Paxinos & Watson rat brain atlas (1997, [18]). The individual creating and analyzing the images was blind to treatment conditions.

For cell counts, regions of interest (ROIs) were first outlined using only the Alexa channel on 10× images. These ROIs included the PVN (AP −1.8, DV −7.7 to −8.2, ML ±0.2 to 0.6) and PFC (AP +3.5, DV −3.0 to −5.0, ML ±0.25 to 1.0). Using the Cy3 channel, individual microglia cells were selected manually and quantified, using the Axiovision 4.6 software.

To quantify the percent area occupied by Iba-1 immunoreactivity, or field density, Zeiss Apotome Deconvolution software was used to take 40× Cy3 Z-stack images. Z-stacks were taken from the medial parvocellular subdivision of the PVN (AP −1.8, DV −7.8 to −8.0, ML ±0.2 to 0.4). In the PFC, Z-stacks were from layers 2 and 3 of the infralimbic cortex (IL), layers 2 and 3 of the prelimbic cortex (PL), layers 5 and 6 of the IL, or layers 5 and 6 of the PL. Using the LSM Image Browser, Z-stacks were merged into several projection images. Each projection image consisted of 5 consecutive 1 μm images from the Z-stack. Using Axiovision automatic program measurements, a consistent threshold level was used to select positive staining and the percent area occupied by the Iba-1 immunoreactivity above threshold was calculated. Objects comprising less than 10 pixels were removed. DyLight lectin labeling was quantified with field density in a similar way. Because the lectin lightly labeled microglial processes, objects 0–300 pixels were removed to ensure that percent area was calculated for vasculature alone. Any extraneous objects were manually removed.

Data analysis was performed using Sigma Stat (Systat Software, San Jose, CA, USA). Data are shown as mean ± SE. Outliers were