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A COMBINED CUMULATIVE THRESHOLD SPECTRA AND DIGITAL RECONSTRUCTION ANALYSIS REVEAL STRUCTURAL ALTERATIONS OF MICROGLIA WITHIN THE PREFRONTAL CORTEX FOLLOWING LOW-DOSE LPS ADMINISTRATION

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Abstract—Sickness behaviors have become the focus of great interest in recent years as they represent a clear case of how peripheral disturbances in immune signaling can disrupt quite complex behaviors. In the current study, we were interested in examining whether we could identify any significant morphological disturbances in microglia associated with these sickness-like behaviors in adult male Sprague–Dawley rats. We chose lipopolysaccharide (LPS 100 μg/kg/i.p.), to induce sickness-like behaviors as it is the most well-validated approach to do so in rodents and humans. We were particularly interested in examining changes in microglia within the prefrontal cortex (PFC) as several recent neuroimaging studies have highlighted significant functional changes in this region following peripheral LPS administration. Paraformaldehyde-fixed tissue was collected from animals 24 h post LPS administration and labeled immunohistochemically with an antibody directed to bind to Iba-1, a protein known to be involved in the structural remodeling of microglia. To analyze changes, we have made use of two recently described image analysis procedures. The first is known as cumulative threshold spectra (CTS) analysis. The second involves the unsupervised digital reconstruction of microglia. We undertook these complementary analysis of microglial cells in the both the pre- and infralimbic divisions of the PFC. Our results indicated that microglial soma size was significantly enlarged, while cell processes had contracted slightly following LPS administration. To our knowledge this study is to first to definitely demonstrate substantial microglial disturbances within the PFC following LPS delivered at a dose that was sufficient to induce significant sickness-like behavior.

Key words: lipopolysaccharide, microglia, prefrontal cortex, sickness behavior.

INTRODUCTION

Almost all mammals suffering from serious viral or bacterial infections have been found to exhibit a set of highly stereotyped behaviors referred to as sickness behaviors. These behaviors include, but are not limited to, reductions in appetite, body weight, fluid intake, locomotor activity, motivation, social interaction, libido, cognitive function and mood state (Hart, 1988). Many have proposed that together these sickness behaviors represent an adaptive response on behalf of the organism to conserve energy, so that all available resources can be redirected toward combating the infection (Hart, 1988).

Perhaps the most common approach to inducing sickness behavior is via the use of a compound known as lipopolysaccharide (LPS) (Yirmiya et al., 1994; Dantzer et al., 2008). LPS represent a large group of molecules that form part of the outer cell wall of gram-negative bacteria (Mancuso et al., 2005). When injected intraperitoneally or intravenously, into otherwise healthy animals or humans, LPS is known to interact with the Toll-like 4 (TLR-4) receptor signaling complex present on monocytes and other leukocytes (Akira and Takeda, 2004). LPS–TLR-4 interaction in turn initiates the release of several pleiotropic immune-signaling molecules including interleukin-1β (IL-1β) and tumor-necrosis factor-α (TNF-α). Sickness behaviors emerge within hours of LPS injection, the appearance of which is tightly correlated with rising levels of circulating IL-1β and TNF-α (Bluthe et al., 2000).

Several research groups have now provided evidence that the emergence of sickness behaviors is critically dependent upon the actions of pro-inflammatory cytokines within the brain. In one of the first studies to demonstrate this Kent delivered IL-1 receptor antagonist (IL-1ra) directly into the brain which effectively quenched the fever and depressive-like effects of IL-1 (Kent et al., 1992a). What remained uncertain for a number of years

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Abbreviations: CTS, cumulative threshold spectra; IL, infralimbic; IL-1β, interleukin-1β; LMA, locomotor activity; LPS, lipopolysaccharide; PBH, phosphate-buffered horse serum; PFC, prefrontal cortex; PL, prelimbic; TLR-4, toll-like 4 receptor; TNF-α, tumor-necrosis factor-α.

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since these early results was specifically how inflammatory signals within the brain were transduced into behavioral disturbances. In relation to this issue, microglia have attracted particular attention for being likely contributors to inflammatory-induced behavioral disturbances. These cells play a central role in both receiving and broadcasting inflammatory signals within the brain and more recently have been shown to directly interact with and modulate synaptic function (Wake et al., 2009; Tremblay et al., 2010; Hinwood et al., 2012; Parkhurst et al., 2013).

Several studies have investigated how peripheral administration of LPS alters microglia *in vivo* and in doing so have made use of one of the two main approaches; thresholding or reconstruction. Thresholding is an image-processing approach that involves manually establishing a particular pixel intensity level (the threshold) that is used to demarcate which pixels in an image are considered to be signal and those which are not (Johnson and Walker, 2015). The number of pixels within the signal range is then quantified and compared across treatment groups. Reconstruction on the other hand, involves creating a manual or automated trace of the immunolabeled microglial cells (usually in two dimensions).

In terms of thresholding-based studies, a single injection of LPS (100 μg/kg, i.p.) has been shown to result in a significant increase in Iba-1 immunoreactivity within the rat paraventricular nuclei and hippocampus (Borges et al., 2012). Radler et al. (2014) has also reported that LPS 50 μg/kg can induce an increase in the intensity of Iba-1 immunoreactive material but in only four of 27 brain regions examined (namely medial preoptic area, subfornical organ, arcuate nucleus and area postrema). Thresholding studies can indicate a change in glial cells but cannot provide information as to the source of this change (whether due to more cells, larger cells, more darkly stained cells, etc.). With respect to reconstruction studies a majority have focused on relatively high doses (1–4 mg/kg). In one of the earliest of these studies, Buttini et al. (1996) demonstrated using high (sepsis-like) doses of LPS (2.5–5 mg/kg) that within 8–24 h microglia lost their highly branched appearance to exhibit an irregular enlarged ameboid appearance. Kozlowski and Weimer (2012) observed similar results in an unspecified region of the cortex following high doses of LPS (1–4 mg/kg). Kloss et al. (2001) in one of the only low-dose examinations of microglial organization, observed that low-to-moderate doses (1–100 μg/kg, i.p.) produced a noticeable enlargement of the cell body and a thickening of proximal processes within microglia of the brainstem. The results from Kloss et al., however, were only observational in nature.

Interestingly, of these studies that investigated how LPS alters microglia none have identified definitive changes in the prefrontal cortex. This is significant as recent work involving the administration of endotoxin to humans has identified that the prefrontal cortex is a particularly sensitive site for changes in functional activation. The prefrontal cortex (PFC) has also consistently been identified as a site that is significantly involved in the regulation of motivational states (O'Reilly, 2010). Microglia in the PFC have been shown to exhibit remarkably consistent morphologies across all layers from medial to lateral (Kongsui et al., 2014b). Moreover, activity within the PFC is known to be disrupted by LPS administration in humans (Kullmann et al., 2013). Accordingly, in the current study we wished to examine if LPS could change microglia within the PFC and specifically within the infralimbic (IL) and prelimbic (PL) divisions of the PFC. Here, instead of using thresholding analysis we have chosen to use a newer approach referred to as cumulative threshold analysis (CTS) as it is known to be both more transparent and robust to differences between groups (Johnson and Walker, 2015). We have complemented this approach by digitally reconstructing cells before and 24 h after injection of 100 μg/kg i.p. of LPS.

EXPERIMENTAL PROCEDURES

Animals

Twenty male Sprague–Dawley rats aged 8 weeks were obtained from the Animal Resource Centre (Perth, Western Australia), and were allowed one week for acclimatization prior to the commencement of experimentation. All animals were single-housed and maintained on a 12:12 reverse light–dark cycle in a constant temperature (21 ± 1 °C) and humidity room with *ad lib* access to food and water. All animals were handed equally, once per day for 7 days prior to commencement of experimental work. All procedures were conducted in accordance with the Newcastle University Animal Care and Ethics Committee guidelines and the New South Wales Animal Research Act and Australian Code of Practice for the use of animals for scientific purposes.

LPS administration

Twenty animals were randomly divided into two groups with half receiving a single intraperitoneal (i.p.) injection of 100 μg/kg of LPS from *Escherichia coli* (strain 0111: B4, Sigma–Aldrich, Osaka, Japan) and the other half receiving 0.9% sterile saline in an injection volume of 1 ml. All animals were handled identically thereafter.

Confirmation of sickness induction post-LPS administration

In order to confirm the efficacy of the LPS administration procedure to induce sickness behavior we chose to monitor food consumption and body weight both prior to and 24 h post LPS administration. Additionally, we assessed the animals' home cage locomotor activity (LMA). Specifically, this involved placing the animals into their home cage (an acrylic box 40 cm (L) × 25 cm (W) × 25 cm (H)) and monitoring them over 24 h using an infrared camera located above the box under low-light conditions (40 Lux). Data were recorded digitally and then analyzed using Ethovision tracking software (Noldus Information Technology, Netherlands) in 5-min increments over the 24-h period. Importantly, an analysis of the recorded tracks indicated that there were no dropouts during the tracking procedure. This absence of dropouts indicates that the animals' *X*/*Y*