Brain, Behavior, and Immunity 26 (2012) 500–510

Contents lists available at SciVerse ScienceDirect

Brain, Behavior, and Immunity

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

Environmental enrichment alters glial antigen expression and neuroimmune function in the adult rat hippocampus

Lauren L. Williamson *, Agnes Chao, Staci D. Bilbo

Department of Psychology & Neuroscience, Duke University, Durham, NC 27708, USA

ARTICLE INFO

Article history:
Received 9 December 2011
Received in revised form 3 January 2012
Accepted 4 January 2012
Available online 20 January 2012

Keywords:
Cytokines
Chemokines
Neurogenesis
Microglia
Astrocytes
Dentate gyrus
Plasticity

ABSTRACT

Neurogenesis is a well-characterized phenomenon within the dentate gyrus (DG) of the adult hippocampus. Environmental enrichment (EE) in rodents increases neurogenesis, enhances cognition, and promotes recovery from injury. However, little is known about the effects of EE on glia (astrocytes and microglia). Given their importance in neural repair, we predicted that EE would modulate glial phenotype and/or function within the hippocampus. Adult male rats were housed either 12 h/day in an enriched environment or in a standard home cage. Rats were injected with BrdU at 1 week, and after 7 weeks, half of the rats from each housing group were injected with lipopolysaccharide (LPS), and cytokine and chemokine expression was assessed within the periphery, hippocampus and cortex. Enriched rats had a markedly blunted pro-inflammatory response to LPS within the hippocampus. Specifically, expression of the chemokines Ccl2, Ccl3 and Cxcl2, several members of the tumor necrosis factor (TNF) family, and the pro-inflammatory cytokine IL-1β were all significantly decreased following LPS administration in EE rats compared to controls. EE did not impact the inflammatory response to LPS in the cortex. Moreover, EE significantly increased both astrocyte (GFAP+) and microglia (Iba1+) antigen expression within the DG, but not in the CA1, CA3, or cortex. Measures of neurogenesis were not impacted by EE (BrdU and DCX staining), although hippocampal BDNF mRNA was significantly increased by EE. This study demonstrates the importance of environmental factors on the function of the immune system specifically within the brain, which can have profound effects on neural function.

1. Introduction

Ongoing adult neurogenesis is now an accepted and well-characterized phenomenon within the mammalian brain, including humans, although its functions have yet to be fully elucidated. In both the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and the subventricular zone of the olfactory bulb, the adult rodent brain regularly produces newly generated cells that can differentiate into neurons, astrocytes and oligodendrocytes. While these stem cells do not differentiate into microglia, it is becoming increasingly evident that microglia are critical for the development and differentiation of neural stem cells (Belmadani et al., 2006; Bhattacharyya et al., 2008; Gonzalez-Perez et al., 2010), and that microglia interact directly with other neural cells throughout normal brain activity (Nimmerjahn et al., 2005; Tremblay and Majewska, 2011; Wake et al., 2009). Moreover, microglia play a key homeostatic role in neuronal survival during the first week after neurons are born in adulthood, by phagocytizing the majority of newborn cells (Sierra et al., 2010). Nonetheless, the role of microglia in neural stem cell plasticity has been almost entirely considered in the context of pathology or disease models that alter neurogenesis (e.g., ischemia, stroke, or immunodeficiency; (for review, see Das and Basu, 2008; Horn and Schlote, 1992; Molina-Holgado and Molina-Holgado, 2010; Pulsinelli et al., 1982; Ziv et al., 2006)). Their role within the normal brain in response to enrichment protocols that augment plasticity has not been fully explored.

Environmental enrichment (EE) is a housing manipulation that increases physical and social stimuli and has been shown to modulate plasticity within the hippocampus and other cortical regions in rodents (Baamonde et al., 2011; Di Garbo et al., 2011; Diamond et al., 1976; Sirevaag and Greenough, 1991). These plasticity changes include increased neurogenesis and cell survival (Kempermann et al., 2002; van Praag et al., 1999), increased gliogenesis (Ehninger and Kempermann, 2003; Steiner et al., 2004), alterations in glial morphology and antigen expression (Viola et al., 2009; Ziv et al., 2006), and the upregulation of growth factors including brain-derived-neurotrophic factor (BDNF), and glial-derived neurotrophic factor (GDNF) (During and Cao, 2006; Ickes et al., 2000; Rossi et al., 2006; Young et al., 1999). Notably, EE is neuroprotective following a number of insults or injuries, including seizures (Steiner et al., 2004; Young et al., 1999), ischemia (Briones et al., 2011), and models of Parkinson's disease (Goldberg et al.,

* Corresponding author. Tel.: +1 919 660 5796; fax: +1 919 660 5798.
E-mail address: llw8@duke.edu (L.L. Williamson).

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doi:10.1016/j.bbi.2012.01.003

2011). The impact of EE on improved neural outcomes following insult is often attributed to increased neurogenesis and/or survival. However, glia (microglia and astrocytes) are the primary immunocompetent cells of the brain, and their function is critical in both injury and repair. The functions of glia in repair may, in fact, be primary underlying mechanisms for improvement or rehabilitation observed after EE.

We examined the impact of 7 weeks of daily EE in rats on neurogenesis and glial alterations within the hippocampus and cortex. We also assessed the cytokine and chemokine response within these regions to a peripheral immune challenge (lipopolysaccharide; LPS) at the end of the EE period. Notably, our enrichment protocol was applied *prior* to inflammatory insult (LPS), in contrast to the majority of studies that first induce injury and thereafter explore the capacity of EE to rescue cell viability or overall function. Our rationale for this approach was to explore the capacity of an environmental change to "buffer" the neuroinflammatory potential of the brain (i.e., prevention rather than rehabilitation). We report that EE specifically alters glial antigen expression within the DG of the hippocampus, and markedly attenuates the immune response to peripheral LPS within the same region without significantly impacting measures of neurogenesis. These changes were specific to the hippocampus, as the glial, neuronal, and cytokine responses within the cortex remained unchanged by EE. Notably, the blunted immune response within the hippocampus of EE rats was specific to a subset of cytokines and chemokines, indicating that these animals were not immunocompromised or deficient. Taken together, the reduced expression of these particular immune molecules may lend insight into the neuroprotective phenotype of EE as well as the unique sensitivity of the hippocampus to inflammatory insult.

2. Materials and methods

2.1. Animals and environmental enrichment

Thirty-two adult (P60) male Sprague–Dawley rats were obtained from Harlan Laboratories (Indianapolis, IN). All rats were pair-housed upon arrival and allowed 1 week to acclimate to the home cage environment. During the course of the experiment, all rats were maintained at 23 °C on a 12:12-h light:dark cycle (lights on at 0700 EST) and given rodent chow and filtered drinking water *ad libitum*. After 1 week, 8 pairs were randomly assigned to environmental enrichment (EE) for a period of 7 weeks for 12 h per day during their dark cycle, while 8 pairs remained in their home cages (HC). This shortened EE paradigm was selected to reflect a more realistic enrichment intervention, in which access to EE would be limited. EE rats were placed in the same enrichment boxes each day at the same time each day. They were also removed from their enriched environments at the same time each day and returned to their home cages. The enrichment boxes (55.9 cm × 35.6 cm × 30.5 cm) contained quarter-inch corn-cob bedding (identical to home cage controls), a running wheel, a PVC tube and various small objects and toys. Enrichment and exercise may impact neurogenesis in the hippocampus differently (Olson et al., 2006); however, our aim was to maximize the effects on neurogenesis with both manipulations. The boxes were cleaned once weekly at the same time as home cage changes. All environments and conducted experiments were approved by the Duke University Institutional Animal Care and Use Committee.

2.2. Administration of BrdU

HC and EE rats were injected intraperitoneally (i.p.) with bromodeoxyuridine (BrdU) (Sigma–Aldrich; 150 mg/kg), once daily for 3 days, beginning one week after the start of daily enrichment. This time point was chosen because previous literature demonstrates that key changes in neurogenesis and alterations in cell survival occur within the first week after plasticity-inducing conditions, such as EE or increased exercise (Dayer et al., 2003; Dobrossy et al., 2003; Epp et al., 2007; Tashiro et al., 2007). Moreover, as mentioned previously, microglia play a key homeostatic role in neuronal survival during the first week after neurons are born in adulthood (Sierra et al., 2010). Enrichment continued for 6 weeks after the first injection.

2.3. Adult immune challenge

After 7 weeks of differential housing and 2 h prior to sacrifice, HC and EE rats were injected i.p. with either sterile saline (SAL) or a 100 μg/kg dose of bacterial lipopolysaccharide (LPS) suspended in sterile saline. These treatments resulted in the four groups shown in this study: HC injected with SAL (HC-SAL), HC injected with LPS (HC-LPS), EE injected with SAL (EE-SAL), and EE injected with LPS (EE-LPS).

2.4. Tissue preparation

Two hours after the SAL or LPS injections, rats were deeply anesthetized with a ketamine/xylazine cocktail. Prior to the start of the perfusion, blood (2 ml) was collected by cardiac puncture and stored on ice until centrifugation. Then rats were perfused transcardially with saline to clear brain vessels of blood. Their brains were extracted; one hemisphere was placed intact into 4% paraformaldehyde solution while the hippocampus and parietal cortex were rapidly dissected from the remaining hemisphere and immediately frozen via immersion in isopentane for later RNA extraction and protein analysis. Intact hemispheres were alternated between post-fixation and dissection and equally distributed across groups. Post-fixed hemispheres were submerged 2 days later in 30% sucrose plus 0.1% sodium azide for cryoprotection and were sliced following submersion. Using a −20 °C cryostat, the intact hemispheres were sliced into forty um coronal sections, taking 5 series of 12 sections each. Sections were stored at 4 °C in a 0.1% sodium azide solution until free-floating immunohistochemistry was performed.

For quantitative real-time PCR, RNA was extracted from hippocampal and adjacent parietal cortex tissue using the Trizol method and DNAse-treated. Complementary DNA (cDNA) was synthesized from 100 ng of isolated RNA for analysis of gene expression using the Qiagen QuantiTect Reverse Transcription Kit (Valencia, CA, USA) or from 500 ng of isolated RNA using the RT^2 First Strand Kit (SABiosciences/Qiagen, Frederick, MD, USA).

2.5. Immunohistochemistry

2.5.1. Basic immunohistochemistry (IHC) protocol

Label-specific antibody differences are noted following this basic protocol.

Free-floating sections were first rinsed for 3 × 5 min in 0.01 M phosphate buffered saline (PBS) and also rinsed before each subsequent step, except between the blocking and primary antibody steps. Next, sections were washed in 50% methanol for 30 min. Sections were then quenched in 0.6% hydrogen peroxide for 30 min and then blocked for another 30 min in 5% normal goat serum and 0.3% Triton-X to block and permeabilize, respectively, in PBS (blocking buffer). Sections were then incubated overnight at room temperature in primary antibody in blocking buffer. The following day, sections were incubated for 2 h at room temperature in a solution of biotinylated secondary antibody in blocking buffer. The Avidin–Biotin Complex (ABC) method was used to bind a complex of streptavidin–biotin peroxidase to the secondary antibody (1 h

incubation), which was then developed with diaminobenzidine (DAB, Vector Laboratories, Burlingame, CA, USA) for 2–15 min to produce a colorimetric stain. Sections were mounted on gel-coated slides, dehydrated and coverslipped with Permount.

2.5.1.1. BrdU. One series (Fig. 1B) of coronal sections were stained with BrdU to quantify all newly dividing cells in the granule cell layer of the dentate gyrus in the hippocampus at the time of BrdU injections. BrdU IHC is similar to the method described above, but includes denaturing steps, which follow the hydrogen peroxide quenching step. Following quenching, sections were incubated at 65 °C in 50:50 formamide:saline-sodium citrate (SSC) solution for 2 h and then rinsed for 2 × 5 min in 2× SSC. Next, sections were incubated in 2 N HCl at 37 °C in a water bath for 30 min followed by a 0.1 M boric acid wash. The sections were then placed into blocking buffer and then the primary antibody buffer overnight (1:300 mouse monoclonal BrdU, Roche Diagnostics, Indianapolis, IN, USA). The next day for secondary antibody incubation, sections were incubated in biotinylated anti-mouse antibody (1:200, Vector Laboratories, Burlingame, CA. USA).

2.5.1.2. DCX. One series (Fig. 1C) of coronal sections was stained with doublecortin (DCX) to identify immature neurons. Normal horse serum replaced normal goat serum for this stain. In the primary antibody step, sections were incubated with DCX antibody (1:200, goat polyclonal, Santa Cruz Biotechnology, Santa Cruz, CA, USA). For the secondary antibody step, sections were incubated in biotinylated anti-goat antibody (1:200, Vector Laboratories, Burlingame, CA, USA).

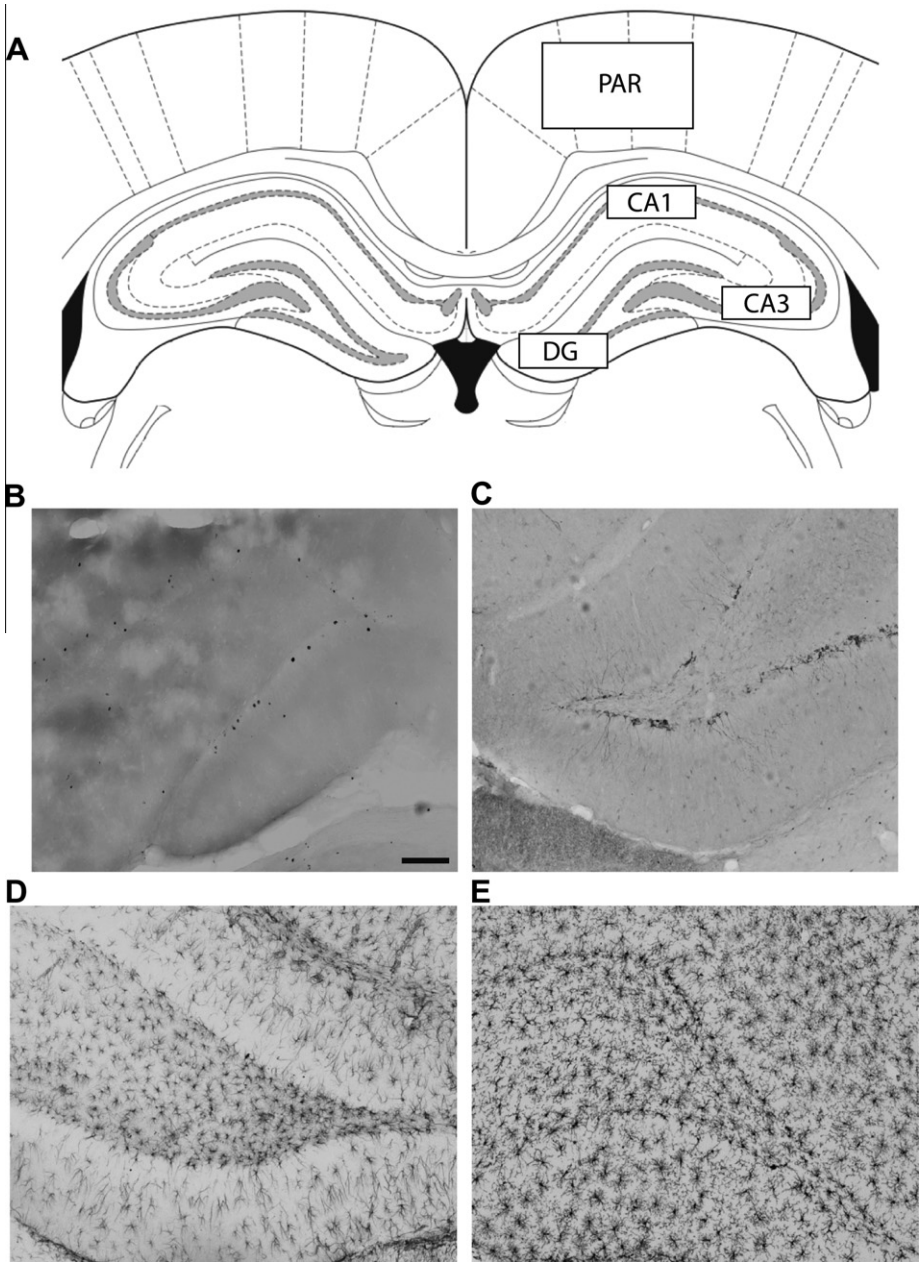

Fig. 1. Regions analyzed for density measures and representative images from immunohistochemical staining. All representative photomicrographs are from the DG of a randomly selected EE rat. (A) Regions of interest for density analysis, (B) bromodeoxyuridine (BrdU), (C) doublecortin (DCX), (D) glial fibrillary acidic protein (GFAP), and (E) ionized calcium binding adaptor molecule 1 (Iba1). Scale bar = 100 um for images B–E.

2.5.1.3. GFAP. One series (Fig. 1D) of coronal sections was stained with glial fibrillary acidic protein (GFAP) to identify astrocytes. In the primary antibody step, sections were incubated with GFAP antibody (1:10,000, mouse monoclonal, Chemicon International, Temecula, CA, USA). For the secondary antibody step, sections were incubated in biotinylated anti-mouse antibody (1:200, Vector Laboratories, Burlingame, CA, USA).

2.5.1.4. Iba1. One series (Fig. 1E) of coronal sections was stained with ionized calcium binding adaptor molecule 1 (Iba1) to identify microglia. For the primary antibody step, sections were incubated in Iba1 antibody (1:10,000, rabbit polyclonal, Wako Pure Chemical Industries, Ltd). In the secondary antibody step, sections were incubated in biotinylated anti-rabbit antibody (1:200, Vector Laboratories, Burlingame, CA, USA).

2.6. Quantification

2.6.1. Cell quantification

Quantification of BrdU-positive cells in the granule cell layer of the dentate gyrus in the hippocampus was performed using a Nikon Eclipse 80i microscope on a Dell PC running StereoInvestigator software (MBF Bioscience, Inc., Williston, VT, USA). The boundaries of the dentate gyrus were traced using this software at a magnification of 10× and BrdU+ cells in the region of interest were counted at 40×. Five sections were counted for each rat and each section was at least 200 μm from the previously counted section. The counts from each individual section were combined to create a single BrdU+ cell count value per rat. Further, BrdU+ counts for rats in the same Housing-Treatment group were averaged to obtain a single BrdU average per group.

2.6.2. Densitometry

Quantification of GFAP-positive and Iba1-positive cells was performed using ScionImage densitometry. Seven sections per rat were analyzed for the dentate gyrus, CA1, and CA3 sub-regions of the hippocampus as well as the parietal cortex (Fig. 1A). Digitized images of each region of interest (ROI) were taken at 20× using a Nikon Eclipse 80i microscope and digital camera on a Dell PC running PictureFrame software. Each region was traced using ScionImage after converting the image to grey scale. Signal pixels of an ROI were defined as having a grey value of 3 SDs above the mean grey value of a cell-poor area close to or within the ROI. The number of pixels and the average grey values above the set background were then computed for each ROI and multiplied by the area of the traced ROI, resulting in an integrated area density measurement. All values for each of the 7 sections per rat were averaged to obtain a single integrated density value per sub-region of interest for each rat. A two-way ANOVA was used to analyze the effects of Housing and Immune Challenge on the individual values obtained for each rat. Integrated density values for rats in the same Housing-Treatment group were averaged to obtain a mean integrated density value per group that is shown on the graphs in Fig. 3.

2.6.3. Quantitative real-time PCR

Gene expression was measured using quantitative real-time PCR with primers designed to measure 85 rat inflammatory cytokines, chemokines and receptors (SABiosciences/Qiagen; Cat. No. PARN-011) or primers listed below using the RT^2 SYBR® Green qPCR Master Mix (Cat. No. PA-010, SABiosciences/Qiagen, Frederick, MD, USA) following the manufacturer's protocol. For the first method, pairs of rats from each treatment group were pooled into 3 samples for a final *n* of 3/treatment group. For the second method, 8 rats/group were analyzed.

2.6.3.1. qRT-PCR analysis. Threshold amplification cycle number (C_t) was determined for each reaction within the linear phase of the amplification plot and relative gene expression was determined using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). Relative gene expression across groups was compared using a two-way ANOVA with housing (home cage or enrichment) and immune challenge (SAL or LPS) as factors. Significant interactions were followed with either the Holm-Sidak or Tukey's post hoc test with an α-level of $p < 0.05$ to determine group differences.

2.6.3.2. Primer specifications. Real-time quantitative PCR primers were obtained from SABiosciences/Qiagen from Cat No. PARN-011 or designed by our laboratory and purchased from Sigma–Aldrich (St. Louis, MO, USA). Sequences: *GAPDH:* Fwd – GTTTGTGAT GGGTGTGAACC, Rev – TCTTCTGAGTGGCAGTGATG; interleukin-1 receptor antagonist (*IL-1ra*): Fwd – GTCTGGAGATGACACCAAG, Rev – TCGGAGCGGATGAAGGTAA; glial-derived neurotrophic factor (*GDNF*): Fwd – ATTCAAGCCACCATCAAAAG, Rev – TCAGTTCCT CCTTGGTTTCG; brain-derived neurotrophic factor (*BDNF, exon 5*): Fwd – ATCCCATGGGTTACACGAAGGAAG, Rev – AGTAAGGGCCCGA ACATACGATTG.

2.7. Protein assessment

2.7.1. Multiplex

A panel of 9 cytokines and chemokines (MCP-1, MIP-1α, IL-1β, IL-10, IFN-γ, IL-18, GRO/KC, Rantes, and TNFα) were measured in serum with a commercially available Luminex bead assay (Millipore Milliplex MAP Kit, Cat. No. RCYTO-80K). Blood from each rat was clotted and centrifuged after collection. The supernatant (serum) was then diluted 5-fold and analyzed according to the manufacturer's instructions. GRO/KC and Rantes were above the detectable range and IL-10 was below the detectable range following these methods; thus, these proteins were not reported.

2.7.2. Corticosterone assessment

Total serum corticosterone concentrations were assessed in serum using a colorimetric EIA kit from Assay Designs, Inc. (Ann Arbor, MI). The assay was run according to the manufacturer's instructions except that serum (5 μl) was diluted 1:50 in 0.05% steroid displacement-modified assay buffer. The detection limit of the assay was 27 pg/ml, and the intra-assay coefficient of variation was 2.03%.

2.8. Statistical analyses

All of the analyses for this study were two-way ANOVAs comparing Housing (HC and EE) and Immune Challenge (SAL and LPS). *F* values for each analysis are reported in Section 3. Significant interactions were followed with either the Holm-Sidak or Tukey's post hoc comparison with an α-level of $p < 0.05$ to determine group differences.

3. Results

3.1. Environmental enrichment increases glial marker density within the DG, whereas markers of neurogenesis remain unchanged

To assess the overall impact of EE on cell genesis/survival within the brain, we measured the expression of BrdU, which labels all newly dividing cells, using immunohistochemistry. The total number of BrdU+ cells within the DG was analyzed as a function of housing and LPS injection using a two-way ANOVA. Surprisingly, there was no effect of EE on total BrdU within the DG 6 weeks after BrdU administration (Fig. 2A). Thus, we also assessed expression of

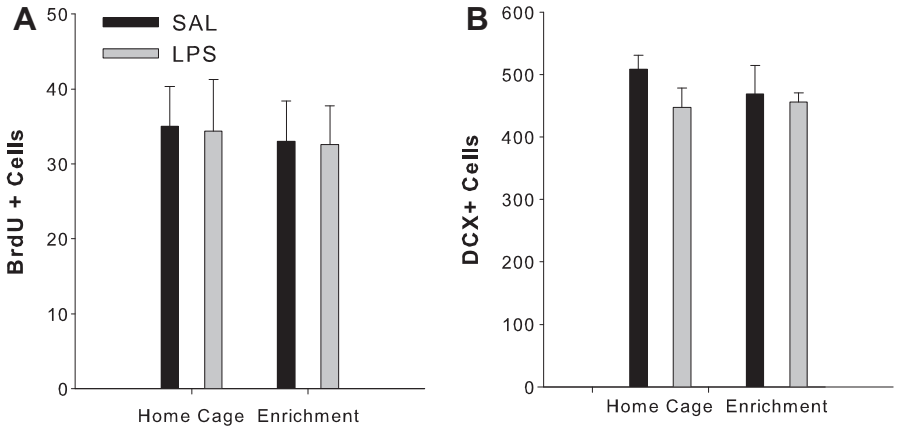

Fig. 2. Daily environmental enrichment for 7 weeks does not increase cell survival or immature neuronal proliferation in the dentate gyrus of the hippocampus. All rats (HC and EE) were given injections of BrdU one week after the beginning of the enrichment paradigm. Six weeks later, all rats were killed 2HR after an LPS injection. Neither BrdU+ cell counts (A) nor doublecortin (DCX+) cells (B) were changed by EE. Values are means ± SEMs of 8 rats/group.

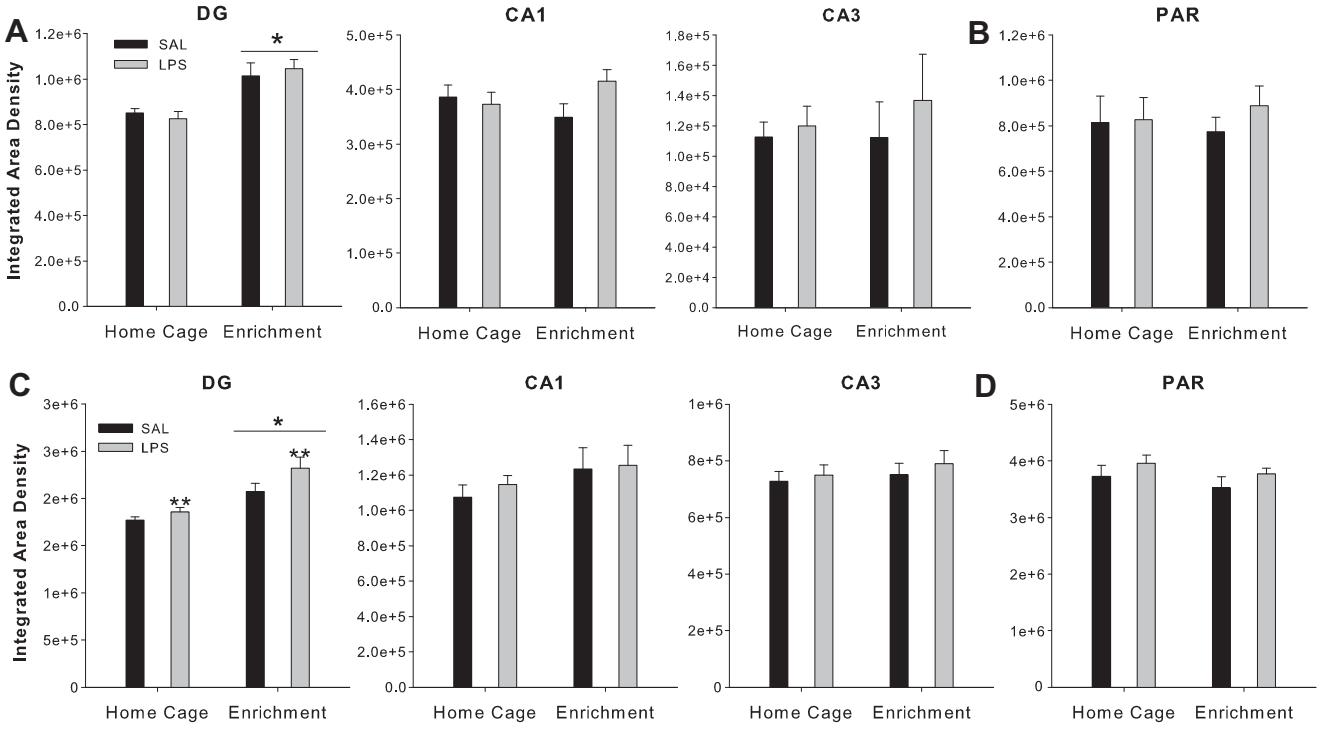

Fig. 3. Environmental enrichment increases glial density in the dentate gyrus (DG), but not in the CA1 or CA3 of the hippocampus or the adjacent parietal cortex. The density of astrocyte marker GFAP (glial fibrillary acidic protein) was increased in the DG of EE rats and not in HC rats (A). In other sub-regions of the hippocampus, CA1 and CA3, GFAP density was similar between the two housing groups (A). Additionally, GFAP density in the parietal cortex adjacent to the hippocampus was similar in each housing group (B). The density of microglial marker Iba1 was increased in the DG of EE rats compared to HC rats (C). Iba1 density in the DG was also increased following an LPS injection and that effect is driven by changes in EE rats. Microglial density was not altered in the CA1 or CA3 regions of the hippocampus (C) or the parietal cortex adjacent to the hippocampus (D). Values are means ± SEMs of 8 rats/group. * Significantly different from home cage, $p < 0.05$ ** Significantly different from SAL, $p < 0.05$.

the immature neuronal marker DCX in adjacent sections. DCX expression is independent of injection time point, a factor that may have contributed to the lack of change in BrdU by EE. However, there were also no significant changes in DCX within the DG following 7 weeks of EE (Fig. 2B). As expected, we did not observe significant BrdU or DCX staining outside the DG (not shown).

To assess the impact of EE on glia, we assessed the expression of the glial antigens GFAP (for astrocytes) and Iba1 (for microglia). In contrast to measures of neurogenesis, there were increases in the density of both GFAP and Iba1 in the EE group. GFAP staining was increased by EE ($F_{(1,28)} = 23.2$, $p < 0.001$) only in the DG sub-region of the hippocampus and was not altered in either the CA1 or CA3 sub-regions (Fig. 2A). Iba1 density in the DG was also significantly increased by EE ($F_{(1,28)} = 23.5$, $p < 0.001$), as well as by LPS treatment ($F_{(1,28)} = 4.45$, $p < 0.05$), an effect predominantly driven by the EE group (Fig. 3C). Similar to GFAP, Iba1 density was not altered in either the CA1 or CA3 sub-regions of the hippocampus. Furthermore, GFAP and Iba1 staining densities were not different in the hippocampal-adjacent parietal cortex across housing and treatment groups (Fig. 3B and D, respectively).

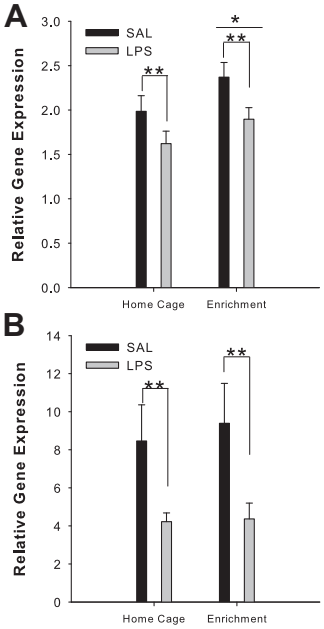

Fig. 4. Environmental enrichment increases brain-derived neurotrophic factor mRNA expression (A), but not glial-derived neurotrophic factor (B) mRNA expression. Both growth factors' expression was significantly reduced by LPS treatment. Values are means ± SEMs of 8 rats/group. * Significantly different from home cage, $p < 0.05$. ** Significantly different from SAL, $p < 0.01$.

3.2. Environmental enrichment increases BDNF within the hippocampus

We assessed the expression of two growth factors within the hippocampus, BDNF and GDNF, which are well described for their roles in neurogenesis and cellular differentiation, especially during environmental enrichment (During and Cao, 2006; Ickes et al., 2000; Rossi et al., 2006; Young et al., 1999). Following 7 weeks of EE, BDNF mRNA was significantly increased within the hippocampus of EE rats ($F_{(1,28)} = 5.7$, $p < 0.05$) (Fig. 4A). In contrast, GDNF mRNA was unaffected by housing ($p > 0.05$) (Fig. 4B). The expression of both growth factors was reduced by LPS (BDNF: $F_{(1,28)} = 8.8$, $p < 0.01$; GDNF: $F_{(1,28)} = 10.4$, $p < 0.01$).

3.3. Environmental enrichment attenuates the hippocampal response to LPS for a subset of cytokines and chemokines

To assess the impact of EE on the central immune response to a peripheral inflammatory challenge, we measured inflammatory cytokine and chemokine mRNA expression within the hippocampus and cortex 2 h following LPS or saline. The pro-inflammatory cytokine interleukin-1β (*IL-1β*) increased within the hippocampus after LPS, but this increase was significantly attenuated in EE rats compared to HC ($F_{(1,8)} = 42.1$, $p < 0.001$) (Fig. 5). Additionally, mRNA expression of several members of the tumor necrosis factor (TNF) pathway was reduced in EE-LPS rats compared to HC-LPS: TNFα (Fig. 6A) ($F_{(1,8)} = 11.3$, $p < 0.05$), the receptor gene TNF receptor super family 1a (*Tnfrsf1a*, Fig. 6B) ($F_{(1,8)} = 5.4$, $p < 0.05$), the receptor gene TNF receptor super family 1b (*Tnfrsf1b*, Fig. 6C) ($F_{(1,8)} = 9.6$, $p < 0.05$), and lymphotoxin alpha (*Lta* or *TNFβ*, Fig. 6D) ($F_{(1,8)} = 17.2$, $p < 0.005$). Finally, the expression of three chemokines known primarily for their functions in leukocyte and monocyte recruitment was reduced in the hippocampus in response to LPS in the EE compared to HC rats: monocyte chemotactic protein 1 (*MCP-1/Ccl2*) ($F_{(1,8)} = 9.4$, $p < 0.05$), macrophage inflammatory protein 1 alpha (*MIP-1α/Ccl3*) ($F_{(1,8)} = 42.1$, $p < 0.001$) and macrophage inflammatory protein 2 alpha (*MIP-2α/Cxcl2*) ($F_{(1,8)} = 10.7$, $p < 0.05$) (Fig. 7). For all genes listed above with the exception of *Lta*, baseline mRNA expression did not differ between saline-treated HC and EE rats.

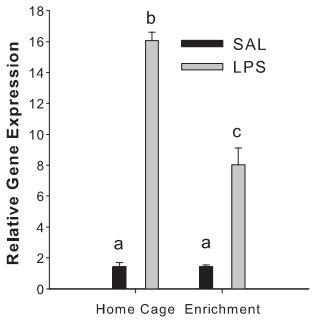

Fig. 5. Environmental enrichment attenuates the interleukin-1β (IL-1β) response in the hippocampus following an LPS challenge. Rats from both housing groups were not different at baseline and responded to the LPS challenge. EE rats had a significantly blunted increase in *IL-1β* mRNA expression following LPS compared to home cage rats. EE-LPS rats had significantly lower IL-1β mRNA expression compared to HC-LPS rats, $p < 0.001$. Values based on means ± SEMs for 3 pairs (6 rats)/group. [a] Baseline value $p > 0.05$. [b] Significantly increased from baseline, $p < 0.05$. [c] Significantly increased from baseline and significantly lower than the HC response, $p < 0.05$.

Further assessment in the hippocampus revealed an extensive list of pro-inflammatory cytokine, chemokine and receptor genes whose transcription increased following LPS treatment (main effect of LPS treatment in 2-way ANOVAs; all p's < 0.05) without alteration by housing (Table 1). In contrast to the hippocampus, all gene expression changes within the parietal cortex adjacent to the hippocampus were due to LPS treatment alone (main effect of LPS treatment in 2-way ANOVAs; all p's < 0.05), without an effect of housing (Table 2). Many of the same genes were upregulated following LPS treatment in both the hippocampus and parietal cortex.

3.4. Environmental enrichment does not impact the peripheral immune or corticosterone response to LPS

We assessed a panel of 9 inflammatory cytokine and chemokine proteins within the serum using a Luminex bead-based assay, to determine whether the impact of EE on the immune response to LPS was specific to brain. Each of the proteins assessed was also measured at the mRNA level within the brain (as discussed previously), though we were only able to measure a small subset of proteins in serum due to the limited availability of rat-specific analytes for the Luminex technology. We did not use Luminex to assess protein expression within *brain* tissue, as this technology has not been validated for rat brain and in our hands generated many high false positives (e.g., on the upper end of detection) without a significant LPS effect (data not shown). In short, we do not believe the measurements are accurate. Our protein assessments in serum showed only an effect of LPS for each protein without an effect of housing. IL-1β (Fig 8A, $p < 0.001$), TNFα (Fig 8B, $p < 0.005$), MCP-1 (Ccl2) (Fig 8C, $p < 0.001$) and MIP-1α (Ccl3) (Fig 8D, $p < 0.001$) were equally increased in the periphery, regardless of housing condition, unlike their responses within the hippocampus. Serum corticosterone, which can markedly impact pro-inflammatory cytokine expression, was also increased by LPS treatment in all rats, without an effect of housing condition ($F_{(1,28)} = 276.3$, $p < 0.001$) (Fig. 9).

4. Discussion

The hippocampus has long been considered to have increased plastic potential compared to other cortical regions, but with its

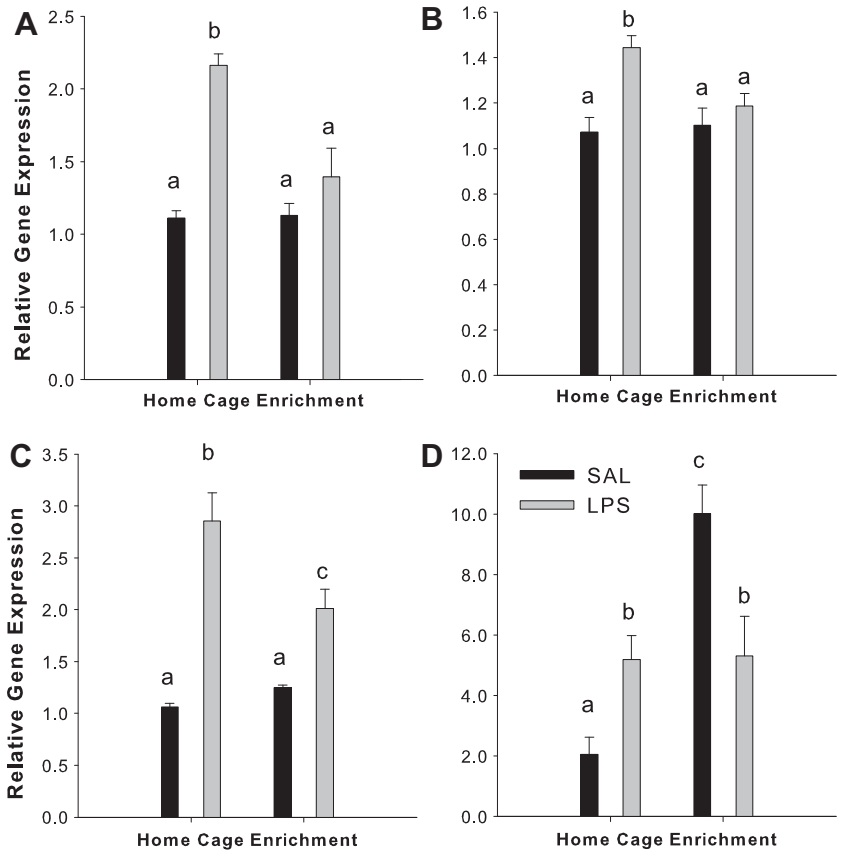

Fig. 6. Environmental enrichment altered the expression of several members of the tumor necrosis factor (TNF) family following an LPS challenge. Tumor necrosis factor alpha (*TNFα*) (A), TNF receptor super family 1a (*Tnfrsf1a*) (B), and TNF receptor super family 1b (*Tnfrsf1b*) (C) had significantly blunted mRNA expression in response to an immune challenge in EE rats. Lymphotoxin alpha (*TNFβ*) (D) was increased at baseline and its expression was reduced in EE rats following LPS. Values based on means ± SEMs for 3 pairs (6 rats)/group. All differences, $p < 0.05$.

increased plasticity may come augmented vulnerability. Cytokine receptors are distributed throughout the brain, but have one of the highest densities in the hippocampus (Cunningham and De Souza, 1993; Schneider et al., 1998). The hippocampus also exhibits marked vulnerability (e.g., cell death and neural dysfunction) relative to other brain regions in response to diverse threats to homeostasis, including stress, epilepsy, stroke, and cardiac arrest (Fujioka et al., 2000; Petito et al., 1987; Salmenpera et al., 1998; Sapolsky et al., 1990). Thus the hippocampus may be particularly sensitive to immune-related alterations (Lynch et al., 2004; Morrison and Hof, 1997). The adjacent parietal cortex that lies dorsal to the hippocampus is responsive to LPS (Williamson et al., 2011); however, the neocortex and hippocampus differ dramatically in structure, connectivity and function. The finding that EE did not affect the neuroimmune response in the parietal cortex, whereas it had a dramatic effect in the hippocampus, underscores the distinction of the hippocampus from other cortical structures.

Environmental enrichment is a well-established protocol for altering the neurogenic niche of the subgranular zone (SGZ) of the DG of the hippocampus. We hypothesized that EE, which induces neuroprotection in a number of studies (Briones et al., 2011; Goldberg et al., 2011; van Praag et al., 2000; Young et al., 1999), would attenuate the neuroinflammatory response to a peripheral immune challenge. Finally, we predicted that EE would impact not only neurogenesis but glia as well, as shown in limited studies (Ziv et al., 2006). Glia are the likely source of the neuroinflammatory cytokines and chemokines of interest, and the impact of EE could potentially alter these signaling molecules. We observed a markedly reduced pro-inflammatory phenotype within the brains of EE rats compared to controls, evidenced by the selective attenuation of a subset of cytokines and chemokines following peripheral LPS. This attenuation was restricted to the hippocampus, and coincident with increased expression of the glial antigens Iba1 and GFAP. Surprisingly, however, these changes were independent of increased neurogenesis, which we did not observe when assessed using BrdU given after 1 week of EE, or with the immature neuronal marker DCX after 7 weeks of EE.

Interestingly, neurogenesis itself is not required for cognitive improvements following enrichment (as shown by Meshi et al., 2006). Our enrichment paradigm was similar in duration by total days to many other studies (for meta-analysis, see Simpson and Kelly, 2011), but few, if any, other studies alter housing *solely* during the dark cycle as we did in this study to more accurately model a "real-world" intervention. It is possible that this paradigm is more stressful than full-time housing; however, we did not observe any significant differences in corticosterone between home cage and EE rats. It is also possible that extending our enrichment paradigm to either full-time housing or 10–12 weeks of limited daily enrichment would increase the population of immature neurons measured by DCX staining, based on prior findings in the field. Our paradigm did, however, elicit other enduring changes in the hippocampi of EE rats. EE increased BDNF mRNA overall within the hippocampus, despite the acute suppression in response to LPS, which are both consistent with the literature (Goshen et al.,

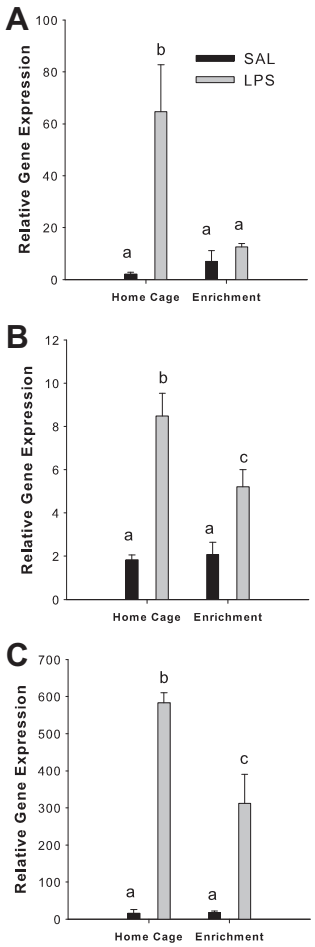

Fig. 7. Following an LPS challenge, environmental enrichment altered the expression of several chemokines – monocyte chemotactic protein 1 (*MCP-1/Ccl2*) (A), macrophage inflammatory protein 1 alpha (*MIP-1α/Ccl3*) (B), and macrophage inflammatory protein 2 alpha (*MIP-2α/Cxcl2*) (C). All 3 chemokines had significantly blunted mRNA expression in EE-LPS rats compared to HC-LPS rats, $p < 0.05$. [a] Baseline value $p > 0.05$. [b] Significantly increased from baseline (a). [c] Significantly increased from baseline and significantly lower than HC response. Values based on means ± SEMs for 3 pairs (6 rats)/group.

2009; Guan and Fang, 2006; Rossi et al., 2006; Schnydrig et al., 2007). Most strikingly, EE increased the density of both Iba1 and GFAP expression specifically within the DG, consistent with a previous report that wheel running exercise increases astrogliogenesis (Ehninger and Kempermann, 2003). Our densitometry assessments do not allow conclusions regarding gliogenesis or number of cells; however, the lack of change in total BrdU strongly argues against an increase in the number of new glia in EE rats. The data instead suggest that EE changes the *phenotype* of glia, altering their activation and attenuating their pro-inflammatory response to peripheral LPS, although this remains to be directly tested. Interestingly, the blunted neuroinflammatory response within the DG of EE rats occurring concomitant with the *increase* in classical glial "activation" markers runs counter to our initial prediction. However, we believe these data simply highlight the fact that little is known about the function of these markers. Moreover, there is a growing literature that distinguishes classical versus alternative activation states in microglia, the latter of which is associated more strongly with repair (Colton, 2009; Colton and Wilcock, 2010). Thus, it is possible that EE shifts microglia into an alternatively activated phenotype, an intriguing possibility that we are currently exploring. Regardless of whether glial number or phenotype is influenced, the notable finding is that there is clearly an impact of EE on glia that occurs without any discernible change in newborn neurons. Importantly, glia were not changed in the CA1 or CA3 sub-regions of the hippocampus, nor were they altered in the adjacent parietal cortex, suggesting that the microenvironment of the DG is conducive to glial changes which may occur independent from neurogenesis. Indeed, glial plasticity may directly interact with and alter neuronal plasticity, questions that remain to be explored.

The immune response within the hippocampi of EE rats was markedly attenuated for a subset of cytokines and chemokines measured in our study. Importantly, not all measured immune molecules were blunted in the hippocampi of EE rats. Furthermore, the immune response was similar for each housing group in the parietal cortex as well as in the periphery. Within the hippocampus, however, EE rats had an attenuated response of interleukin-1β (IL-1β), the TNF family of genes, and several chemokines involved in the recruitment of leukocytes and monocytes. These families of genes indicate an altered hippocampal milieu in EE rats that may be less pro-inflammatory, more neuroprotective and less permeable to peripheral infiltrating immune cells.

As several studies have shown, IL-1β is a pro-inflammatory molecule that is also important for normal brain function (Cibelli et al., 2010; Goshen et al., 2009; Goshen et al., 2007; Rachal Pugh et al., 2001; Tanaka et al., 2011; Yirmiya and Goshen, 2011). Importantly, physiological levels of IL-1β expression are critical for normal learning and memory (Goshen et al., 2007), whereas levels that are either too low or too high become detrimental (Williamson et al., 2011). In the present study, EE rats have a blunted IL-1β response to an LPS dose that elicits a robust increase in mRNA expression in HC controls. Numerous other studies have demonstrated improved learning and memory performance in EE rats (Iuvone et al., 1996; Kulesskaya et al., 2011; Kumar et al., 2011; Meshi et al., 2006; Pacteau et al., 1989; Pham et al., 1997; Simpson

Table 1
Chemokines and cytokines in the hippocampus altered by treatment with LPS, regardless of housing condition. All *p*-values < 0.05. Values based on means ± SEMs for 3 pairs (6 rats)/group.

Gene	Reference ID	HC-SAL	HC-LPS	EE-SAL	EE-LPS	Main effect of LPS
Ccl22	NM_057203	1.6 ± 0.3	4.4 ± 1.2	1.2 ± 0.1	5.4 ± 0.3	$F_{1,8} = 32.2$, $p < 0.001$
Ccl7	NM_001007612	4.6 ± 1.8	42.1 ± 15.7	5.3 ± 2.7	13.8 ± 1.9	$F_{1,8} = 8.1$, $p < 0.022$
Cxcl1	NM_030845	4.2 ± 0.4	82.0 ± 20.1	2.6 ± 0.8	41.5 ± 4.0	$F_{1,8} = 32.4$, $p < 0.001$
Cxcl9	NM_145672	1.7 ± 0.1	27.3 ± 3.5	1.4 ± 0.2	26.6 ± 3.8	$F_{1,8} = 96.4$, $p < 0.001$
Cxcl10	NM_139089	6.1 ± 0.7	1061.2 ± 149.7	7.7 ± 3.6	915.5 ± 153.3	$F_{1,8} = 83.9$, $p < 0.001$
Cxcl11	NM_182952	2.5 ± 0.3	603.4 ± 148.3	3.1 ± 1.4	381.9 ± 59.8	$F_{1,8} = 37.5$, $p < 0.001$
IL-1a	NM_017019	3.1 ± 0.4	1.7 ± 0.4	3.5 ± 0.1	1.7 ± 0.4	$F_{1,8} = 19.5$, $p = 0.002$
IL-1ra	NM_022194	2.8 ± 0.4	6.4 ± 1.5	3.3 ± 0.8	6.5 ± 1.8	$F_{1,28} = 10.4$, $p = 0.003$
IL2rg	NM_080889	1.3 ± 0.2	7.9 ± 0.9	1.3 ± 0.1	6.13 ± 0.2	$F_{1,8} = 145.9$, $p < 0.001$
IL6ra	NM_017020	1.3 ± 0.1	2.4 ± 0.1	1.2 ± 0.1	2.0 ± 0.2	$F_{1,8} = 52.1$, $p < 0.001$
IL8rb	NM_017183	2.3 ± 0.8	9.2 ± 4.6	1.6 ± 0.3	7.4 ± 1.9	$F_{1,8} = 6.1$, $p = 0.039$
IL-15	NM_013129	1.5 ± 0.1	1.3 ± 0.2	1.6 ± 0.1	1.1 ± 0.1	$F_{1,8} = 5.7$, $p = 0.044$

Table 2
Chemokines and cytokines in the parietal cortex altered by treatment with LPS, regardless of housing condition. All p-values < 0.05. Values based on means ± SEMs for 3 pairs (6 rats)/group.

Gene	Reference ID	HC-SAL	HC-LPS	EE-SAL	EE-LPS	Main effect of LPS
Bcl6	XM_221333	1.1 ± 0.1	1.6 ± 0.1	1.2 ± 0.02	1.9±0.2	$F_{1,8} = 39.5$, $p < 0.001$
Ccl3	NM_013025	1.4 ± 0.2	4.9 ± 0.5	2.1 ± 0.4	4.2 ± 0.3	$F_{1,8} = 62.7$, $p < 0.001$
Ccl5	NM_031116	1.4 ± 0.3	2.4 ± 0.5	1.6 ± 0.2	2.5 ± 0.4	$F_{1,8} = 6.0$, $p = 0.040$
Cxcl1	NM_030845	1.3 ± 0.3	10.9 ± 3.2	1.3 ± 0.02	10.5 ± 0.8	$F_{1,8} = 32.1$, $p < 0.001$
Cxcl2	NM_053647	1.8 ± 0.2	62.8 ± 20.2	1.8 ± 0.7	40.0 ± 6.2	$F_{1,8} = 21.9$, $p = 0.002$
Cxcl9	NM_145672	1.6 ± 0.3	91.0 ± 31.1	2.3 ± 0.3	81.8 ± 9.6	$F_{1,8} = 26.8$, $p < 0.001$
Cxcl10	NM_139089	2.0 ± 0.7	208.8 ± 50.0	1.7 ± 0.3	205.8 ± 13.5	$F_{1,8} = 63.0$, $p < 0.001$
Cxcl11	NM_182952	3.4 ± 0.9	549.3 ± 233.8	1.7 ± 0.5	405.2 ± 49.1	$F_{1,8} = 15.8$, $p = 0.004$
Cxcr5	NM_053303	1.2 ± 0.3	3.8 ± 1.0	1.4 ± 0.2	3.5 ± 0.7	$F_{1,8} = 13.5$, $p = 0.006$
IL-1b	NM_031512	2.6 ± 0.4	29.5 ± 10.3	2.3 ± 0.8	19.4 ± 3.7	$F_{1,8} = 15.9$, $p = 0.004$
IL1r2	NM_053953	1.4 ± 0.4	3.0 ± 0.6	1.7 ± 0.2	2.2 ± 0.1	$F_{1,8} = 8.4$, $p = 0.02$
IL2rg	NM_080889	1.5 ± 0.2	7.2 ± 1.0	1.1 ± 0.04	6.1 ± 0.4	$F_{1,8} = 95.0$, $p < 0.001$
IL6ra	NM_017020	1.1 ± 0.04	2.6 ± 0.3	1.2 ± 0.03	2.2 ± 0.2	$F_{1,8} = 44.2$, $p < 0.001$
IL8ra	NM_019310	3.0 ± 0.9	6.6 ± 1.1	2.2 ± 0.8	7.0 ± 0.6	$F_{1,8} = 23.5$, $p = 0.001$
IL8rb	NM_017183	2.1 ± 0.8	7.5 ± 1.8	1.5 ± 0.3	6.4 ± 1.3	$F_{1,8} = 18.9$, $p = 0.002$
IL-11	NM_133519	1.3 ± 0.2	2.3 ± 0.3	1.5 ± 0.1	1.9 ± 0.3	$F_{1,8} = 9.7$, $p = 0.014$
IL-15	NM_013129	1.6 ± 0.03	1.2 ± 0.1	2.0 ± 0.1	1.3 ± 0.2	$F_{1,8} = 13.7$, $p = 0.006$
Lta	NM_080769	2.1 ± 0.5	3.4 ± 0.4	1.6 ± 0.6	2.6 ± 0.4	$F_{1,8} = 5.5$, $p = 0.046$
Tnfrsf1b	NM_130426	1.3 ± 0.2	2.9 ± 0.4	1.3 ± 0.1	2.4 ± 0.1	$F_{1,8} = 43.5$, $p < 0.001$

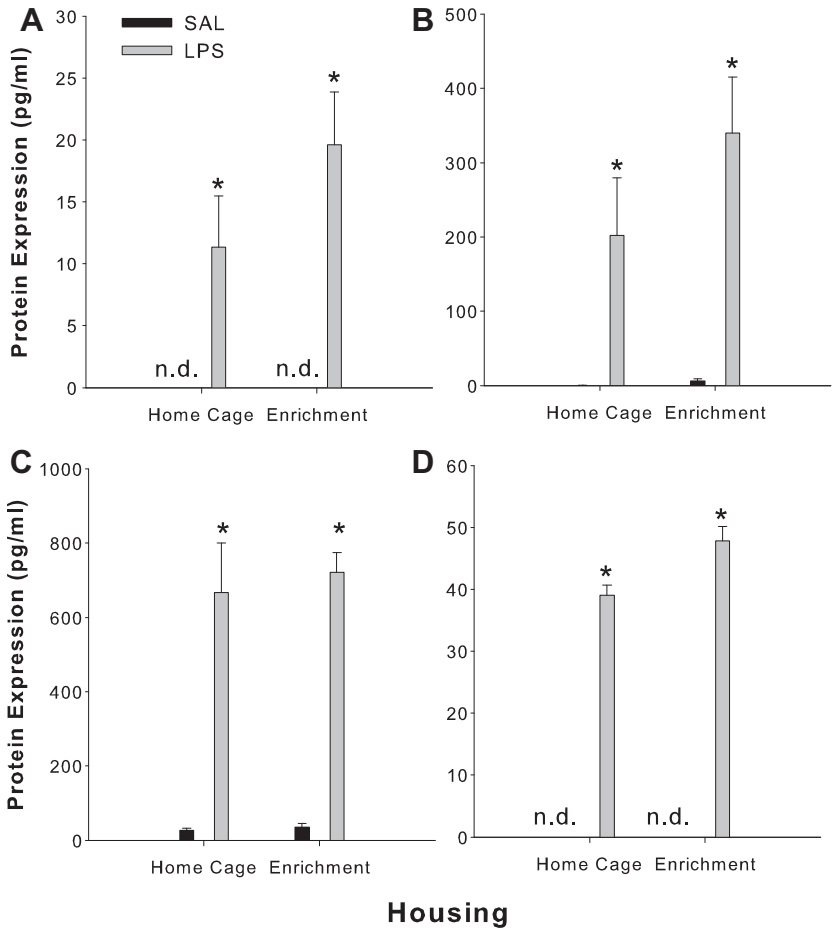

Fig. 8. In the periphery, LPS treatment significantly increased protein expression in the serum of the following cytokines and chemokines: interleukin-1β (*IL-1β*) (A), tumor necrosis factor alpha (TNFα) (B), monocyte chemotactic protein 1 (*MCP-1*/*Ccl2*) (C), and macrophage inflammatory protein 1 alpha (*MIP-1α*/*Ccl3*) (D). Values are means ± SEMs of 8 rats/group. * Significantly different than SAL, $p < 0.005$.

and Kelly, 2011). Notably, the source of IL-1β during hippocampal-dependent learning, as well as during an immune response within the hippocampus, is solely CD11b+ microglia (Williamson et al., 2011). Altered microglial reactivity in response to EE may therefore have significant consequences for learning and memory processes, especially in the context of infection or illness, which remains to be explored. Similarly, many studies use EE as a rehabilitation paradigm in a diseased brain model (e.g., Parkinson's disease, (Goldberg

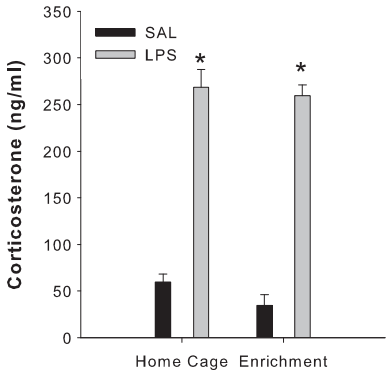

Fig. 9. An immune challenge with LPS significantly increased serum corticosterone, regardless of housing group. Baseline corticosterone was not altered by housing. Values are means ± SEMs of 8 rats/group. * Significantly different from SAL, $p < 0.001$.

et al., 2011)); in these studies, IL-1β tone may exceed optimal levels in the inflamed, diseased brains without an EE intervention.

Tumor necrosis factor alpha (TNFα) is well characterized for its roles in inflammation and host defense, sepsis and, most intriguing for this study, apoptosis cascades (for review, see Hehlgans and Pfeffer, 2005). The observed attenuation after an immune challenge of TNFα and several associated genes in EE rats compared to HC controls indicates a potential enduring change in the hippocampal microenvironment of enriched rats, such that one mechanism by which EE may increase neuroprotection following insults to the CNS (Briones et al., 2011; Goldberg et al., 2011; Young et al., 1999) is via altered TNF tone and function, increasing the likelihood of cell survival by reducing apoptotic signaling. In addition to attenuated IL-1β and TNF responses, EE rats showed blunted responses for several chemokines known to influence the recruitment of circulating monocytes and leukocytes to the CNS. Monocyte chemoattractant protein 1 (MCP-1/CCL2) is important for recruitment of peripheral monocytes into the brain during an immune challenge or infection (e.g. HIV; Lee et al., 2011). Following status epilepticus (SE) in rats, triggered by stimulation of the amygdala, a paradigm known to increase aberrant neurogenesis and neuronal injury in the hippocampus, macrophage inflammatory protein 1-alpha (MIP-1α/CCL3) is increased for up to 30 days (Guzik-Kornacka et al., 2011). Taken together, the attenuated expression of these cytokines and chemokines in EE rats treated with LPS demonstrate possible mechanisms for neuroprotective and increased plasticity outcomes associated with EE.

In summary, environmental enrichment is a relatively simple manipulation that results in robust beneficial outcomes for the brain. While previous studies have shown a role in post-insult rehabilitation for EE, our study provides evidence that enrichment need not follow the insult in order to be beneficial. Indeed, neuroinflammatory disease states might be attenuated or delayed in their onset in the face of ongoing EE. The translational reach of this manipulation remains to be explored, but in animal models of neuroinflammation, EE may provide a simple preventative measure for negative outcomes. Furthermore, this study presents further evidence that EE alters microglia and astrocytes within the DG of the hippocampus. Though the mechanism remains to be investigated, these changes in glia in the DG may underlie the attenuated immune response in the brains of EE rats treated with LPS, and these same glial changes may be directly related to neural changes often associated with EE. As an increasing number of studies explore the effects of glia (both microglia and astrocytes) on normal brain function, manipulations that alter both glia and neurons alike may provide perspective on interactions between these cell types as well as their specific roles in the healthy brain.

Acknowledgements

The authors would like to acknowledge the technical assistance of Emily Ngan. LLW is supported by a National Science Foundation Graduate Research Fellowship. This work was made possible by R01 MH083698. The authors declare no conflict of interest.

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