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Acute Stress Induces Chronic Neuroinflammatory, Microglial and Behavioral Priming: A Role for Potentiated NLRP3 Inflammasome Activation

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Abstract

Prior exposure to acute and chronic stressors potentiates the neuroinflammatory and microglial pro-inflammatory response to subsequent immune challenges suggesting that stressors sensitize or prime microglia. Stress-induced priming of the NLRP3 inflammasome has been implicated in this priming phenomenon, however the duration/persistence of these effects has not been investigated. In the present study, we examined whether exposure to a single acute stressor (inescapable tailshock) induced a protracted priming of the NLRP3 inflammasome as well as the neuroinflammatory, behavioral and microglial proinflammatory response to a subsequent immune challenge in hippocampus. In male Sprague-Dawley rats, acute stress potentiated the neuroinflammatory response (IL-1 β , IL-6, and NF κ B α) to an immune challenge (lipopolysaccharide; LPS) administered 8 days after stressor exposure. Acute stress also potentiated the proinflammatory cytokine response (IL-1 β , IL-6, TNF and NF κ B α) to LPS *ex vivo*. This stress-induced priming of microglia also was observed 28 days post-stress. Furthermore, challenge with LPS reduced juvenile social exploration, but not sucrose preference, in animals exposed to stress 8 days prior to immune challenge. Exposure to acute stress also increased basal mRNA levels of NLRP3 and potentiated LPS-induction of caspase-1 mRNA and protein activity 8 days after stress.

The present findings suggest that acute stress produces a protracted vulnerability to the neuroinflammatory effects of subsequent immune challenges, thereby increasing risk for stress-related psychiatric disorders with an etiological inflammatory component.

Further, these findings suggest the unique possibility that acute stress might induce innate immune memory in microglia.

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Keywords

stress; neuroinflammation; inflammasome; NLRP3; priming; microglia

1. Introduction

Prior exposure to acute and chronic stressors potentiates the neuroinflammatory and microglial pro-inflammatory response to subsequent immune challenges (de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Frank et al., 2007; Frank et al., 2018; Frank et al., 2012; Johnson et al., 2003; Johnson et al., 2004; Munhoz et al., 2006; Wohleb et al., 2011), suggesting that stressors sensitize or prime microglia, which are considered a key substrate of this stress-induced phenomenon (Frank et al., 2015).

A number of studies suggest that activation of the NLRP3 (NOD-, LRR- and pyrin domain-containing protein 3) inflammasome plays a pivotal role in mediating these priming effects of stress (Alcocer-Gomez et al., 2015; Bharti et al., 2019; Feng et al., 2019; Iwata et al., 2015; Pan et al., 2014; Weber et al., 2015). The NLRP3 inflammasome is an intra-cellular multiprotein complex that forms in response to a diverse array of damage-associated molecular patterns (DAMPs) as well as microbial-associated molecular patterns (MAMPs) (Elliott and Sutterwala, 2015; Lamkanfi and Kanneganti, 2010; Swanson et al., 2019). Generally considered, the NLRP3 inflammasome consists of a sensor unit (oligomerized NLRP3), an adaptor protein (ASC; apoptosis-associated speck-like protein containing a caspase-recruitment domain) and an effector protein (caspase-1). Unlike the activation of other inflammasomes, NLRP3 inflammasome formation and activation requires a 2-step process. The first, or priming step, typically involves the ligation of Toll-Like Receptors (TLRs), which bind DAMPs and/or MAMPs leading to NLRP3 gene transcription, translation and protein production (Swanson et al., 2019). Once sufficient NLRP3 protein has been formed and the cell is “primed”, a second activation signal triggers oligomerization of NLRP3, which recruits the adaptor protein ASC, which then recruits pro-caspase-1 through its caspase recruitment domain. This assembly of proteins is considered the inflammasome, and once formed, triggers the activation/cleavage of pro-caspase 1. Mature, active caspase-1 then acts to cleave pro-IL-1 β (and pro-IL-18) into their mature biologically active forms. This 2-step of process of NLRP3 inflammasome activation is considered the canonical pathway, however a non-canonical pathway of activation has also been characterized, which does not require a priming step (Swanson et al., 2019).

Our prior work suggests that acute stress-induced NLRP3 inflammasome priming plays a pivotal role in neuroinflammatory and microglia priming (Weber et al., 2015); however the duration/persistence of these effects has not been investigated. In the present study, we examined whether exposure to single acute stressor induces a protracted priming of the NLRP3 inflammasome as well as priming of the neuroinflammatory, microglial and behavioral response to a subsequent immune challenge.

2. Methods

2.1. Subjects

Male Sprague-Dawley rats (225–250 g; Envigo, Indianapolis, IN) were pair housed on a 12-h light-dark cycle (lights on at 0700 h). Food (standard laboratory chow) and water were available *ad libitum*. Rats were allowed to acclimate to colony conditions for at least one week prior to experimentation. All experiments were approved by the Institutional Animal Care and Use Committee of the University of Colorado Boulder in compliance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* as well as the ARRIVE guidelines for animal use.

2.2. Inescapable tailshock (IS)

Details of the stressor protocol have been published previously and this protocol reliably potentiates pro-inflammatory cytokine responses in the hippocampus after peripheral immune challenge (Johnson et al., 2003) as well as in isolated hippocampal microglia to LPS *ex vivo* (Frank et al., 2007). Briefly, animals were placed in Plexiglas tubes (23.4 cm in length $\times$ 7 cm in diameter) and exposed to 100–1.6 mA, 5 s tail-shocks with a variable inter-trial interval (ITI) ranging from 30 – 90 s (average ITI = 60 s). All IS treatments occurred between 09:00 and 11:00 h. Home cage control (HCC) animals remained undisturbed in their home cages.

2.3. In vivo immune challenge

Eight days after IS exposure, IS and HCC animals were injected IP with vehicle (0.9% saline) or lipopolysaccharide (LPS; 10 μ g/kg). LPS (*E. coli* serotype 0111: B4; Sigma, L3012) was dissolved in pyrogen free, sterile 0.9% saline. 2 h after injection, all animals were anesthetized, saline perfused and brain tissue collected for mRNA and protein analysis.

2.4. Tissue dissection of hippocampus

Animals were given a lethal dose of sodium pentobarbital. Animals were fully anesthetized and transcardially perfused with ice-cold saline (0.9%) for 3 min to remove peripheral immune leukocytes from the CNS vasculature. Brain was rapidly extracted and hippocampus dissected. Hippocampus was flash frozen in liquid nitrogen for whole tissue analysis. For microglia *ex vivo* studies, hippocampus was immediately processed. All tissue samples were stored at -80°C . We chose hippocampus as a focus of our studies given the robust effects of stress on neuroinflammatory and microglial priming in this region (Frank et al., 2007).

2.5. Tissue processing for protein assays

Hippocampal samples were sonicated on ice using a tissue extraction reagent (Invitrogen, FNN0071) supplemented with a protease inhibitor cocktail (Sigma-Aldrich, P2714). Homogenates were centrifuged ($14,000 \times g$ for 10 min at 4°C) and supernatants collected and stored at -80°C . Total protein was quantified using a Bradford assay.

2.6. Enzyme-linked immunosorbent assay (ELISA)

An ELISA for rat IL-1 β (R&D Systems, RLB00) was run according to the manufacturer's instructions and protein levels normalized to total protein.

2.7. Caspase-1 activity assay

Caspase-1 activity was measured using the Caspase-Glo[®] Inflammasome Assay (Promega, G9951). According to the manufacturer's instructions, the Z-WEHD-aminoluciferin substrate plus the proteasome inhibitor (MG-132), which blocks non-specific cleavage of the Z-WEHD-aminoluciferin substrate, was added to Caspase-Glo[®] 1 buffer. WEHD (Trp-Glu-His-Asp) substrates are tetrapeptides that exhibit the highest sensitivity to the proteolytic activity of a number of caspases including caspases 1 and 11 (Poreba et al., 2013). The caspase-1 assay used here incorporates a selective and irreversible caspase-1 inhibitor (Ac-YVAD-CHO) (Thornberry et al., 1992), which is used to determine caspase-1 specific activity apart from total caspase activity. Thus, Ac-YVAD-CHO was added to the Z-WEHD-aminoluciferin substrate buffer to yield 2 substrates: Z-WEHD-aminoluciferin without Ac-YVAD-CHO and Z-WEHD-aminoluciferin including Ac-YVAD-CHO.

For each sample, protein homogenate (25 μ l) was added to two wells of a 96 well white-coated plate (Costar, 3917). One well received Z-WEHD-aminoluciferin substrate (25 μ l) buffer minus Ac-YVAD-CHO, while the second well received Z-WEHD-aminoluciferin substrate (25 μ l) buffer plus Ac-YVAD-CHO resulting in a Z-WEHD concentration of 20 μ M and an Ac-YVAD-CHO concentration of 1 μ M. Luminescence of the Z-WEHD-aminoluciferin substrate was measured at 15, 30 and 45 min of incubation at 37 °C using a Tecan Infinite M200 Pro plate reader (Männedorf, Switzerland). Caspase-1 activity was quantified according to the following formula: total relative luminescence units (RLU)(–Ac-YVAD-CHO) - residual RLU (+Ac-YVAD-CHO) = caspase-1 specific RLU. Data are expressed as RLU/total protein.

2.8. Ex vivo immune stimulation of hippocampal microglia with LPS

Eight days after IS exposure, hippocampal microglia were isolated using a Percoll (Sigma, P1644) density gradient as previously described (Frank et al., 2006). This procedure of isolating cells takes ~1.5h. We have previously shown (Frank et al., 2006) that this microglia isolation procedure yields highly pure microglia (Iba-1+/CD163–/GFAP–). In the present experiments, immunophenotype and purity of microglia was assessed using real time RT-PCR. Microglia were suspended in DMEM+10% FBS and microglia concentration determined by trypan blue exclusion. Microglia concentration was adjusted to a density of 1×10^4 cells/100 μ l and 100 μ l added to individual wells of a 96-well v-bottom plate. LPS was utilized to challenge microglia *ex vivo* as we have previously determined the optimal *in vitro* conditions under which LPS stimulates a microglia pro-inflammatory cytokine response (Frank et al., 2006). Cells were incubated with LPS (1, 10, and 100 ng/ml) or media alone for 2 h at 37°C, 5% CO₂. The plate was centrifuged at $1000 \times g$ for 10 min at 4°C to pellet cells and cells washed 1x in ice cold PBS and centrifuged at $1000 \times g$ for 10 min at 4°C. Cell lysis/homogenization and cDNA synthesis was performed according to the manufacturer's protocol using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen, 18080–200).

2.9. Real time RT-PCR measurement of gene expression

Total RNA was isolated from hippocampus using TRI Reagent (MilliPore Sigma, 93289) and a standard method of phenol:chloroform extraction (Chomczynski and Sacchi, 1987). Total RNA was quantified using a NanoDrop 2000 spectrophotometer (ThermoFisher). cDNA synthesis was performed using the SuperScript II Reverse Transcriptase kit (ThermoFisher, 18064014). A detailed description of the PCR amplification protocol has been published previously (Frank et al., 2006). cDNA sequences were obtained from Genbank at the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov). Primer sequences were designed using the Operon Oligo Analysis Tool (<http://www.operon.com/tools/oligo-analysis-tool.aspx>) and tested for sequence specificity using the Basic Local Alignment Search Tool at NCBI (Altschul et al., 1997). Primers were obtained from ThermoFisher. Primer specificity was verified by melt curve analyses. All primers were designed to span exon/exon boundaries and thus exclude amplification of genomic DNA. Primer sequences are detailed in Table 1. PCR amplification of cDNA was performed using the Quantitect SYBR Green PCR Kit (Qiagen, 204145). Formation of PCR product was monitored in real time using the CFX96 Touch Real-Time PCR Detection System (BioRad). Relative gene expression was determined using *Actb* as the housekeeping gene and the 2^{-CT} method (Livak and Schmittgen, 2001).

2.10. Juvenile Social Exploration (JSE)

JSE is a widely used measure of social avoidance and validated measure of anxiety (File and Seth, 2003) and is sensitive to the neuroinflammatory effects of stress and immunogenic agents such as LPS (Goshen and Yirmiya, 2009). Here, JSE was measured 2 days prior to (baseline) and 2h, 6h and 24h post-LPS/vehicle treatment. Each experimental rat was transferred to a novel cage with shaved wood bedding in a dimly lit room (40 lx). After a 15-min habituation period, a 28–32 day-old juvenile male rat was introduced to the subject's cage for 5 min. Exploratory behaviors of the adult (sniffing, pinning, licking and allo-grooming of the juvenile) were timed by an observer blind to treatment condition. After the test, the juvenile was removed and the experimental adult rat was returned to its home cage. Although juvenile stimulus rats were reused for multiple tests, the adult was never re-tested with the same juvenile. For each animal, JSE test data were quantified as a percent of baseline JSE.

2.11. Sucrose preference

To assess behavioral anhedonia, rats were provided with two bottles, one containing water and one containing water supplemented with 2% sucrose (side of sucrose bottle was counterbalanced within groups). Rats were acclimated to the two-choice test for 4 h on the night directly prior to baseline assessment. Sucrose intake was then measured for 4 h each night at baseline and following the experimental manipulations (24 and 48h post-LPS or vehicle injection) and a percentage of relative sucrose intake was calculated: sucrose intake/(sucrose intake + water intake) $\times$ 100.

2.12. Statistical analysis

All data are presented as mean $\pm$ sem. Statistical analyses consisted of t-test or ANOVA followed by post-hoc tests (Tukey's HSD) using Prism 8 (Graphpad Software, LLC). In several instances, data is scaled to the mean of the HCC animals and presented as a percent of the HCC mean. Here, the mean of the HCC group was computed and all individual data points for a particular analyte were divided by the HCC mean * 100, which sets the HCC mean at 100% with a specific standard error. For microglia *ex vivo* experiments, area under the LPS concentration curve (AUC) was computed to capture the cumulative effect of stress on the cytokine response to LPS *ex vivo*. This transformation of the raw data results in a cumulative cytokine response measure for each animal as a function of stress treatment. Threshold for statistical significance was set at $\alpha = 0.05$. Sample sizes are provided in figure captions.

3. Results

3.1. Neuroinflammatory priming

Initially, we examined whether the phenomenon of stress-induced priming of the neuroinflammatory response persisted 8 days after exposure to an acute stressor (100 inescapable tailshocks; IS). Here, animals were exposed to IS and 8 days after stressor exposure, animals were administered LPS (10 μ g/kg), which served as an immune challenge. Indeed, we found that exposure to IS 8 days prior to immune challenge potentiated the hippocampal pro-inflammatory cytokine response to LPS (Fig. 1) including IL-1 β mRNA (interaction, $F(1,28) = 14.67$, $p < 0.001$), IL-6 mRNA (interaction, $F(1,28) = 24.78$, $p < 0.0001$), NF κ B α mRNA (interaction, $F(1,26) = 27.01$, $p < 0.0001$) and IL-1 β protein (interaction, $F(1,26) = 11.4$, $p = 0.002$). IS failed to potentiate the effect of LPS on TNF mRNA, however the main effect of LPS was significant ($F(1,28) = 5.16$, $p = 0.03$). Given that stress potentiated the neuroinflammatory response to LPS 8 days after stressor exposure, we examined whether a similar priming effect of stress occurs in hippocampal microglia.

3.2. Hippocampal microglial priming

Here, hippocampal microglia were isolated 8 days after stressor exposure and treated with several concentrations of LPS *ex vivo* to determine if prior stress potentiated the microglial proinflammatory response. We found that exposure to IS potentiated the hippocampal microglia response to LPS (Fig. 2) for IL-1 β mRNA ($t(14) = 7.33$, $p < 0.0001$), IL-6 mRNA ($t(14) = 5.51$, $p < 0.0001$), NF κ B α , mRNA ($t(14) = 5.24$, $p = 0.0001$) and the TNF mRNA ($t(14) = 5.22$, $p = 0.0001$) response to LPS. To further characterize the extent to which prior stressor exposure produces a protracted priming of microglia proinflammatory responses, we examined stress-induced microglia priming 28 days post-stress. In this study, we exposed hippocampal microglia to a single concentration of LPS (100 ng/ml) *ex vivo* given that the magnitude of priming is greatest at this concentration at 8 days post-IS. At this timepoint post-IS, the microglia proinflammatory cytokine response to LPS for IL-1 β mRNA (interaction effect, $F(1,6) = 48.73$, $p = 0.0004$) and TNF mRNA (interaction effect, $F(1,6) = 42.43$, $p = 0.0006$) was still potentiated by stress compared to the response observed in HCCs (Fig. 3). The effect of stress on IL-6 mRNA and NF κ B α , mRNA was not significant (data not shown).

3.3. Sickness behavior priming

The stress-induced potentiated pro-inflammatory cytokine response observed *in vivo* and *ex vivo* suggests that cytokine-sensitive sickness behaviors might also be potentiated by prior stressor exposure. Therefore, we examined whether prior stressor exposure might potentiate reductions in social exploration and sucrose preference elicited by LPS treatment administered 8 days after IS exposure.

3.3.1. Social exploration (Fig. 4)—The social exploration test (social avoidance) is considered a behavioral measure of anxiety (File and Seth, 2003) and is sensitive to the effects of stress (Christianson et al., 2008) as well as immune challenge such as LPS (Fonken et al., 2018). At 2 h post-LPS treatment, prior IS exposure potentiated the effect of LPS on reductions in social exploration (interaction effect, $F(1,28) = 9.64$, $p = 0.0043$). LPS treatment in IS animals resulted in a significant reduction in social exploration compared to vehicle-treated IS animals ($p < 0.001$), vehicle-treated HCCs ($p < 0.05$) and LPS-treated HCCs ($p < 0.05$). At 6h post-LPS, a similar effect of IS on LPS-induced reductions on social exploration was observed (interaction effect, $F(1,26) = 11.75$, $p = 0.002$). LPS treatment in IS animals resulted in a significant reduction in social exploration compared to vehicle-treated IS animals ($p < 0.001$), vehicle-treated HCCs ($p < 0.01$) and LPS-treated HCCs ($p < 0.01$). Of note, LPS failed to reduce social exploration in HCCs compared to vehicle-treated HCCs and IS exposed animals at both the 2 h and 6 h timepoint post-LPS treatment. A consideration here is that this lack of an LPS effect in HCCs might have been due to missed injections and/or tolerance to LPS. However, we examined LPS-induced weight loss in all experimental groups and found that LPS significantly reduced body weight in both HCCs and IS exposed animals compared to vehicle controls (main effect of LPS, $F(1, 28) = 24.61$, $p < 0.001$). Body weight did not significantly differ between LPS-treated HCCs and LPS-treated IS exposed animals. At 24 h post-LPS treatment, prior IS exposure failed to potentiate the effect of LPS on reductions in social exploration.

3.3.2. Sucrose preference—To further characterize the effect of stress on LPS-induced sickness behaviors, we examined preference for sucrose, which is considered a behavioral measure of anhedonia (Willner et al., 1987). Eight days after stress exposure, sucrose preference was measured 1 day prior (baseline) and 24 h and 48 h after LPS administration. Baseline sucrose preference did not significantly differ between experimental groups (data not shown). Stress failed to potentiate the LPS-induced reductions in sucrose preference at 24 h (interaction effect, $F(1, 12) = 0.008$, $p = 0.93$) and 48 h (interaction effect, $F(1, 12) = 0.33$, $p = 0.57$) post-LPS administration. However, LPS independent of stress significantly reduced sucrose preference at 24 h (main effect, $F(1, 12) = 6.89$, $p = 0.02$), but not at 48 h (main effect, $F(1, 12) = 2.46$, $p = 0.14$) post-LPS (data not shown).

3.4. NLRP3 inflammasome

As noted in the Introduction, a number of studies suggest that priming of the NLRP3 inflammasome might play a role in stress-induced neuroinflammatory and microglia priming to subsequent immune challenges (Alcocer-Gomez et al., 2015; Bharti et al., 2019; Feng et al., 2019; Iwata et al., 2015; Pan et al., 2014; Weber et al., 2015). Further, the NLRP3 inflammasome is the only inflammasome that requires a priming step (Latz et al., 2013). A

number of inflammasomes, apart from NLRP3, have been characterized including NLRP1, AIM2 (absent in melanoma 2) and NLRC4 (NLR Family CARD Domain containing 4), which drive caspase-1 activation, but do not require a priming step for activation to occur (Broz and Dixit, 2016). Therefore, in light of this unique priming property of the NLRP3 inflammasome, we explored the possibility that the NLRP3 inflammasome remains primed 8 days after stress exposure.

3.4.1. Effect of stress on basal NLRP3 mRNA—NLRP3 inflammasome priming requires increased expression of NLRP3 mRNA (Swanson et al., 2019), therefore we examined NLRP3 gene expression in hippocampus and hippocampal microglia 8 days after exposure to IS (Fig. 5). We found that IS increased NLRP3 gene expression 8 days post-IS ($t(10) = 3.18$, $p = 0.01$). In addition, we examined whether IS induced prolonged increases in the basal gene expression of a number of alternate genes involved in neuroinflammatory processes. These genes included IL-1 β , IL-6, TNF, NF κ B α , HMGB1, TLR4, and RAGE. Prior exposure to IS failed to alter basal expression of these genes 8 days after stress except for NF κ B α which was significantly increased compared to HCCs ($t(10) = 2.85$, $p = 0.02$). Of note, NF κ B α expression is driven by NF κ B (Sun et al., 1993), which is also a key upregulator of NLRP3 gene expression (Jo et al., 2016). We also examined the effect of stress on NLRP3 and NF κ B α expression in hippocampal microglia and found that prior IS resulted in a protracted upregulation of NLRP3 ($t(14) = 2.46$, $p = 0.03$) as well as NF κ B α ($t(14) = 2.91$, $p = 0.01$) expression compared to HCCs.

3.4.2. Effect of stress on basal mRNA levels of alternate inflammasome genes—In light of the effect of IS on NLRP3 gene expression, we examined whether prior IS exposure upregulated basal gene expression of alternate inflammasomes including NLRP1, AIM2 and NLRC4 (Fig. 6). Prior IS failed to upregulate the basal expression of NLRP1 ($t(14) = 0.065$, $p = 0.95$) and AIM2 ($t(14) = 0.02$, $p = 0.99$), while for NLRC4, prior IS downregulated expression ($t(14) = 2.6$, $p = 0.02$) 8 days after stressor exposure compared to HCCs.

3.4.3. Effect of stress on LPS induction of NLRP3 mRNA—Here, we examined whether prior stress exposure might potentiate the effect of LPS on NLRP3 gene expression both *in vivo* and *ex vivo*. We found that prior IS failed to potentiate the effect of LPS on NLRP3 in hippocampus (Fig. 7) (interaction effect, $F(1,28) = 0.16$, $p = 0.69$), however IS did upregulate expression of NLRP3 independent of LPS treatment compared to HCCs (main effect, $F(1,28) = 5.61$, $p = 0.025$). However, in hippocampal microglia (Fig. 8), prior IS did potentiate the effect of LPS on NLRP3 *ex vivo* 8 days after stress ($t(14) = 5.00$, $p = 0.0002$) as well as 28 days after stress (interaction effect, $F(1, 12) = 13.96$, $p = 0.003$).

3.4.4. Effect of stress on LPS induction of the NLRP3 inflammasome adaptor ASC and effector caspase-1—As noted, the NLRP3 inflammasome consists of an NLRP3 oligomer, the adaptor protein ASC and the effector protein caspase-1 (Swanson et al., 2019), which functions as a holoenzyme to process pro-IL-1 β into its mature, biologically active form (Boucher et al., 2018). Therefore, we examined the effect of prior IS on LPS induction of ASC mRNA, caspase-1 mRNA and caspase-1 protein activity 8 days

after IS (Fig. 9). Prior exposure to IS differentially altered the effect of LPS on ASC gene expression (interaction effect, $F(1, 28) = 12.9$, $p = 0.0012$). LPS resulted in a significant downregulation of ASC expression in HCCs as compared to vehicle-treated HCC animals ($p < 0.05$), vehicle-treated IS animals ($p < 0.01$) and LPS-treated IS animals ($p < 0.0001$) suggesting that prior IS prevented the LPS-induced downregulation of ASC. For caspase-1 mRNA, prior IS potentiated the effect of LPS (interaction effect, $F(1,28) = 27.39$, $p < 0.0001$) compared to vehicle-treated HCCs ($p < 0.0001$), vehicle-treated IS animals ($p < 0.0001$) and LPS-treated HCCs ($p < 0.0001$). Given this effect of IS on LPS-induction of caspase-1 mRNA, we examined whether prior IS might potentiate LPS-induction of caspase-1 protein activity.

Towards addressing this priming effect of IS, we utilized a caspase-1 activity assay to measure activity in protein homogenates of hippocampus. As this assay has not, to our knowledge, been utilized previously to assess caspase-1 activity in brain homogenates, we conducted a number of experiments *in vitro* to optimize and validate this assay. Utilizing an active form of rat recombinant caspase-1, we found that the assay detected caspase-1 activity in a concentration and time-dependent manner (Suppl. Fig. S1). That is, caspase-1 cleavage of the substrate (Z-WEHD-aminoluciferin) was blocked in the presence of the selective caspase-1 inhibitor Ac-YVAD-CHO. In hippocampal protein homogenates, we also found that cleavage of the substrate was optimal at 37°C and that maximal cleavage occurred at 30–40 min of incubation at this temperature (data not shown). It is important to note that the substrate used here (Z-WEHD-aminoluciferin) to detect caspase-1 activity might also serve as a substrate for other inflammatory caspases, in particular caspase-11 (Poreba et al., 2013). Indeed, we found that recombinant caspase-11 cleaved Z-WEHD-aminoluciferin in the absence of the caspase-1 inhibitor Ac-YVAD-CHO (Suppl. Fig. S2). However, in the presence of Ac-YVAD-CHO, caspase-11 activity was not blocked, but actually potentiated in the presence of Ac-YVAD-CHO. Nonetheless, these findings suggest that this assay is highly selective for caspase-1 activity. Given these findings, we assessed hippocampal caspase-1 activity 8 days after IS and found that prior IS potentiated the effect of LPS on caspase-1 activity (interaction effect, $F(1,28) = 7.78$, $p < 0.01$) compared to vehicle-treated HCCs ($p < 0.001$), vehicle-treated IS animals ($p < 0.01$) and LPS-treated HCCs ($p < 0.001$).

4. Discussion

The present set of findings suggest that prior exposure to a single stressor session produces a protracted priming of the neuroinflammatory, microglial proinflammatory and behavioral sickness response to subsequent immune challenge. Our prior work examining this priming phenomenon of stress was restricted to only examining timepoints of immune challenge proximal to stressor exposure (i.e., 24 h post-stress) (Frank et al., 2007). Here, we sought to determine the duration/persistence of this priming phenomenon because, if an acute stressor produces a chronic vulnerability to the neuroinflammatory effects of subsequent immune challenges, this might increase risk for stress-related psychiatric disorders with an etiological inflammatory component (Miller and Raison, 2016). Here, we found that exposure to acute stress potentiated the hippocampal neuroinflammatory response (IL-1 β , IL-6, and NF κ B α) to an immune challenge (LPS) administered 8 days after stressor exposure. Measurement of the neuroinflammatory response was restricted to a small set of

proinflammatory mediators (IL-1 β , IL-6, TNF and NF κ B1 α) given their prominent role in the sickness response to stress (Goshen and Yirmiya, 2009) and immune challenge (McCusker and Kelley, 2013). It is important to consider that a number of studies have also found that chronic stress also primes the neuroinflammatory response to a subsequent immune challenge (de Pablos et al., 2014; de Pablos et al., 2006; Espinosa-Oliva et al., 2011; Franklin et al., 2018; Munhoz et al., 2006; Wang et al., 2017; Wohleb et al., 2012; Wohleb et al., 2011). However, these studies largely examined the priming effects of immune challenge within 24 h after termination of the stress regimen. Of note, Franklin et al. examined immune markers of priming (RAGE and HMGB1 expression) 28 d after termination of chronic stress and found both markers upregulated in brain (Franklin et al., 2018) suggesting that chronic stressors might also induce a protracted priming of the neuroinflammatory response to immune challenges.

In the CNS, microglia are a key source of inflammatory mediators (Kettenmann et al., 2011) under a variety of inflammatory conditions including stress exposure (Frank et al., 2019). We have previously found that prior stress exposure potentiates the proinflammatory response of hippocampal microglia *ex vivo* 24 h after stress (Frank et al., 2007). Thus, we examined whether microglial priming might still be present 8 days after stress exposure. Indeed, prior stress potentiated the proinflammatory cytokine response (IL-1 β , IL-6, TNF and NF κ B1 α) to LPS *ex vivo*. Interestingly, the magnitude of microglial priming at 8 days post-stress was similar to the magnitude of priming observed in our earlier studies (Frank et al., 2007), in which priming was assessed 24 h after stressor exposure. This finding suggests that the magnitude of priming induced by prior stress is maintained for at least 1 week after stress exposure. Given this finding, we examined the possibility that stress-induced priming of microglia might last considerably longer than 1 week post-stress. Thus, we chose to examine microglia priming 28 days after stress exposure and found that microglia still exhibited a primed phenotype at this distal time point post-stress. However, it should be noted that the magnitude of priming at 28 days post-stress was less than the magnitude observed at 8 days post-stress and was limited to IL-1 β and TNF. Nevertheless, this finding suggests that acute stress can produce a protracted priming of microglia, which is characterized by high durability.

As noted, proinflammatory cytokines such as IL-1 β play a causal role in the sickness response to stress and immune challenge (Dantzer, 2009). Thus, given the neuroinflammatory priming effects observed 8 days after stress exposure, we examined whether prior stress might potentiate the sickness response to LPS administered 8 days after stress. Two behaviors were examined, JSE and sucrose preference, both of which are sensitive to the effects of proinflammatory cytokines. We found that challenge with LPS reduced JSE only in animals exposed to stress 8 days prior to immune challenge. It is important to note that LPS failed to significantly reduce JSE in home cage control animals, which might be due to the low dose of LPS (10 μ g/kg) used here. In referring to Figure 1, it is notable that LPS failed to significantly increase IL-1 β and IL-6 mRNA as well as IL-1 β protein in home cage control animals. However, LPS did significantly reduce body weight in all home cage control animals, suggesting that all home cage controls had received and responded physiologically to LPS. Thus, we can exclude the possibility that the observed stress-induced priming effects, whether neuroinflammatory or behavioral, were simply due

to LPS non-responses in controls. The dose of LPS used here is considered a sub-threshold dose, which allows detection of the priming effects of prior stress, but elicits minimal neuroinflammatory effects independent of stress. With regard to sucrose preference, prior stress exposure failed to potentiate the LPS-induced decrements in preference, however LPS independent of stress did suppress sucrose preference at 24 h post-LPS, although the effect was marginal. Examining preference at timepoints more proximal to LPS administration (e.g. 2 h) might have revealed stress-induced priming effects.

As noted, the NLRP3 inflammasome has been implicated in the neuroinflammatory effects of stress as well as the priming effects of stress (Alcocer-Gomez et al., 2015; Bharti et al., 2019; Feng et al., 2019; Iwata et al., 2015; Pan et al., 2014; Weber et al., 2015). Of particular relevance to the phenomenon of stress-induced priming, the NLRP3 inflammasome is the only inflammasome that requires a priming step as part of a two-step process for activation to occur (i.e., caspase-1 activation and maturation of IL-1 β protein)(Swanson et al., 2019). Thus, the NLRP3 inflammasome is uniquely situated to mediate stress-induced priming phenomena. Therefore, we examined whether stress exposure might produce a protracted priming of the NLRP3 inflammasome given the priming effects of prior stressor exposure on the neuroinflammatory, behavioral and microglial responses to LPS 8 days after stress. Increased mRNA levels of NLRP3 is considered a requisite component of NLRP3 priming (Swanson et al., 2019). Therefore, we examined NLRP3 gene expression in hippocampus and hippocampal microglia 8 days post-stress. Indeed, prior stress increased NLRP3 expression in both hippocampus and hippocampal microglia. This finding is consistent with our prior finding that acute stress increases NLRP3 expression (Weber et al., 2015) as well as Iwata and colleagues who also found that 1 h of immobilization stress increases activation of the NLRP3 inflammasome (Iwata et al., 2015). Chronic stress exposure also produces increases in NLRP3 expression (Feng et al., 2019; Pan et al., 2014). Again, it is important to note that these studies examined NLRP3 expression within 24 h after termination of the stressor. In light of this effect of stress on NLRP3, we determined if this effect of stress extended to other inflammasomes (NLRP1, AIM2 and NLRC4), which do not require a priming step to be activated (Netea et al., 2015). We found that prior stress failed to increase expression of these alternate inflammasomes. In addition to increasing NLRP3 gene expression, prior stress exposure also increased expression of NF κ B α , which is a surrogate for NF κ B signaling (Sun et al., 1993). Of note, NF κ B signaling upregulates NLRP3 gene expression (Jo et al., 2016). Interestingly, chronic stress exposure also upregulates phospho-NF κ B expression in tandem with upregulating NLRP3 expression (Feng et al., 2019; Pan et al., 2014). Given that prior stress appears to prime the NLRP3 inflammasome, we examined whether prior stress might potentiate the NLRP3 response to LPS *in vivo* and *ex vivo*. While prior stress failed to potentiate the effect of LPS on NLRP3 *in vivo*, stress did potentiate the effect of LPS *in vitro* 8 and 28 days post-stress. However, stress did upregulate NLRP3 gene expression independent of LPS *in vivo*. Finally, we determined whether prior stress might upregulate other components of the NLRP3 inflammasome including the adaptor protein ASC and the effector protein caspase-1. We found that prior stress prevented the LPS-induced down-regulation of ASC. In addition, prior stress potentiated the LPS-induced increase in caspase-1 mRNA and protein activity. Of note, LPS failed to increase caspase-1 mRNA and protein activity in home cage controls, which is consistent with the lack of an

LPS effect on IL-1 β protein (See Figure 1D). Again, this might be due to the low dose of LPS used here. Taken together, these effects of prior stress on components of the NLRP3 inflammasome suggest that prior stress produces a protracted priming of the NLRP3 inflammasome.

4.1. Limitations

Several key limitations to this study should be noted. First, it is unclear whether the stress-induced priming of NLRP3 inflammasome activation plays a causal role in stress-induced priming of the neuroinflammatory, behavioral and microglial proinflammatory response to subsequent immune challenge. As noted above, apart from NLRP3, several inflammasomes including NLRP1, AIM2 and NLRC4 are capable of activating caspase-1 and maturation of IL-1 β (Netea et al., 2015). Therefore, given the correlative nature of the present study, it is unclear which inflammasome(s) mediates the priming effects of stress. Future studies using pharmacological blockade of NLRP3 activation (e.g. MCC-950) are required to address a causal role for NLRP3. Second, the present study was restricted to examining priming effects in microglia. Interestingly, microglia regulate hippocampal neurogenesis (Ekdahl et al., 2003; Ekdahl et al., 2009; Monje et al., 2003) and neuroinflammatory processes are considered detrimental to adult neurogenesis, which is thought to underpin the pivotal role of microglia in stress-induced depressive-like behavior (Yirmiya et al., 2015). Therefore, it would be of interest to examine the protracted priming effects of stress on hippocampal neurogenesis. In addition, neuroinflammatory processes are clearly not limited to microglia, but can also involve astrocytes (Colombo and Farina, 2016; Michalovicz et al., 2019), which have not been explored in the priming effects of stress. This avenue of investigation into astrocytes raises intriguing possibilities. Finally, the present findings raise a key question as to the mechanism whereby exposure to a single acute stressor produces protracted priming of the NLRP3 inflammasome. One potential mechanism might involve stress-induced epigenetic alterations in the NLRP3 promoter, which might permit persistent access to transcription factors (i.e., NF κ B) that drive NLRP3 transcriptional upregulation. Notably, epigenetic changes in innate immune genes due to inflammatory insults have been characterized and underlie long-term functional reprogramming of these genes. This phenomenon of protracted functional reprogramming has been termed innate immune memory (Dominguez-Andres and Netea, 2019).

4.2. Conclusions

The present study suggests that acute stress produces a protracted vulnerability to the neuroinflammatory effects of subsequent immune challenges, thereby increasing risk for stress-related psychiatric disorders with an etiological inflammatory component (Miller and Raison, 2016). Further, these findings suggest the unique possibility that acute stress might induce innate immune memory in microglia.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

1. Acute stress produces protracted priming of hippocampal microglia.
2. Acute stress produces protract priming of the NLRP3 inflammasome.
3. Acute stress-induced priming might reflect innate immune memory in microglia.

Figure 1. Effect of prior stress on LPS-induced neuroinflammatory processes.

Animals were exposed to IS or served as HCCs. Eight days after stressor exposure, animals were administered vehicle or LPS (10 μ g/kg, IP). Two hours post-injection, hippocampal proinflammatory cytokine mRNA was measured for (A) IL-1 β (***) $p < 0.001$, IS/LPS vs IS/vehicle, HCC/LPS and HCC/vehicle), (B) IL-6 (***) $p < 0.001$, IS/LPS vs IS/vehicle, HCC/LPS and HCC/vehicle), (C) NF κ B mRNA (**** $p < 0.0001$, IS/LPS vs IS/vehicle, HCC/LPS and HCC/vehicle; ** $p < 0.01$, HCC/LPS vs HCC/vehicle and IS/vehicle) and (D) TNF (** $p < 0.01$, IS/LPS vs IS/vehicle, HCC/LPS and HCC/vehicle). Data are presented as the mean + sem; N = 6–8 per experimental group; RFU = relative fluorescence units; HCC = home cage control; IS = inescapable shock; LPS = lipopolysaccharide.

Figure 2. Effect of prior stress on LPS-induced proinflammatory processes in hippocampal microglia.

Animals were exposed to IS or served as HCCs. Eight days after stressor exposure, hippocampal microglia were isolated, treated with LPS (0, 1, 10 and 100 ng/ml) for 2h and proinflammatory cytokine mRNA measured. Panels **A** (IL-1β), **C** (IL-6), **E** (NFκBα) and **G** (TNF) represent the cytokine response to various concentrations of LPS. Panels **B** (IL-1β, *** $p < 0.001$, IS vs HCC), **D** (IL-6, *** $p < 0.001$, IS vs HCC), **F** (NFκBα, *** $p < 0.001$, IS vs HCC) and **H** (TNF, *** $p < 0.001$, IS vs HCC) represent area under the LPS concentration curve (AUC) for each cytokine, which was computed to capture the cumulative effect of stress on the cytokine response to LPS *ex vivo*. Data are presented as the mean + sem; N = 8 per experimental group; RFU = relative fluorescence units; HCC = home cage control; IS = inescapable shock; LPS = lipopolysaccharide.

Figure 3. Effect of stress on LPS-induced proinflammatory processes in hippocampal microglia 28 days after stressor exposure.

Animals were exposed to IS or served as HCCs. Twenty-eight days after stressor exposure, hippocampal microglia were isolated, treated with LPS (0 and 100 ng/ml) for 2h and proinflammatory cytokine mRNA measured for (A) IL-1 β (***) $p < 0.0001$, IS/LPS vs IS/vehicle, HCC/LPS and HCC/vehicle) and (B) TNF (***) $p < 0.001$, IS/LPS vs IS/vehicle, HCC/LPS and HCC/vehicle). Data are presented as the mean + sem; N = 4 per experimental

group; RFU = relative fluorescence units; HCC = home cage control; IS = inescapable shock; LPS = lipopolysaccharide.

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Figure 4. Effect of prior stress on LPS-induced reductions in juvenile social exploration (JSE). Animals were exposed to IS or served as HCCs. Eight days after stressor exposure, animals were administered vehicle or LPS (10 ug/kg, IP). JSE was measured at (A) 2h (* $p < 0.05$, IS/LPS vs HCC/vehicle and HCC/LPS; *** $p < 0.001$, IS/LPS vs IS/vehicle), (B) 6h (** $p < 0.01$, IS/LPS vs HCC/vehicle and HCC/LPS; *** $p < 0.001$, IS/LPS vs IS/vehicle) and (C) 24h post-injection. Data are presented as the mean + sem; $N = 6-8$ per experimental group; RFU = relative fluorescence units; HCC = home cage control; IS = inescapable shock; LPS = lipopolysaccharide.

Figure 5. Effect of prior stress on basal NLRP3 and NFκB1α gene expression.

Animals were exposed to IS or served as HCCs. Eight days after stressor exposure, NLRP3 gene expression (**A**: hippocampus, * $p < 0.05$, IS vs HCC; **B**: hippocampal microglia, * $p < 0.05$, IS vs HCC) and NFκB1α gene expression (**C**: hippocampus (* $p < 0.05$, IS vs HCC; **D**: hippocampal microglia, * $p < 0.05$, IS vs HCC) was measured. Data are presented as the mean + sem; N = 6 per experimental group; RFU = relative fluorescence units; HCC = home cage control; IS = inescapable shock.

Figure 6. Effect of prior stress on basal gene expression of alternate inflammasomes.

Animals were exposed to IS or served as HCCs. Eight days after stressor exposure, (A) NLRP1, (B) AIM2 and (C) NLRC4 (* $p < 0.05$, IS vs HCC) gene expression was measured in hippocampus. Data are presented as the mean + sem; $N = 8$ per experimental group; RFU = relative fluorescence units; HCC = home cage control; IS = inescapable shock.

Figure 7. Effect of prior stress on LPS-induced NLRP3 gene expression.

Animals were exposed to IS or served as HCCs. Eight days after stressor exposure, animals were administered vehicle or LPS (10 ug/kg, IP). Two hours post-injection, hippocampal NLRP3 mRNA was measured (* $p < 0.05$, IS vs HCC). Data are presented as the mean + sem; N = 8 per experimental group; RFU = relative fluorescence units; HCC = home cage control; IS = inescapable shock; LPS = lipopolysaccharide.

Figure 8. Effect of prior stress on LPS-induced NLRP3 in hippocampal microglia.

Animals were exposed to IS or served as HCCs. **(A)** Eight days after stressor exposure, hippocampal microglia were isolated, treated with LPS (0, 1, 10 and 100 ng/ml) for 2h and NLRP3 mRNA measured. Inset represents area under the LPS concentration curve (AUC) for NLRP3, which was computed to capture the cumulative effect of stress on the cytokine response to LPS *ex vivo* (***) $p < 0.001$, IS vs HCC). **(B)** Twenty-eight days after stressor exposure, hippocampal microglia were isolated, treated with LPS (0 and 100 ng/ml) for 2h and NLRP3 mRNA measured (***) $p < 0.001$, IS/LPS vs IS/media, HCC/LPS and HCC/media). Data are presented as the mean + sem; **(A)** $N = 8$ and **(B)** $N = 4$ per experimental group; RFU = relative fluorescence units; HCC = home cage control; IS = inescapable shock; LPS = lipopolysaccharide.

Figure 9. Effect of prior stress on the LPS-induced NLRP3 inflammasome adaptor (ASC) and effector (caspase-1). Animals were exposed to IS or served as HCCs.

Eight days after stressor exposure, animals were administered vehicle or LPS (10 ug/kg, IP). Two hours post-injection, hippocampal (A) ASC gene expression (* $p < 0.05$, HCC/LPS vs HCC/vehicle; ** $p < 0.01$, HCC/LPS vs vehicle/IS; *** $p < 0.001$, HCC/LPS vs IS/LPS), (B) caspase-1 gene expression (*** $p < 0.001$, IS/LPS vs HCC/vehicle, HCC/LPS and IS/vehicle) and (C) caspase-1 enzymatic activity (*** $p < 0.001$, IS/LPS vs HCC/vehicle, HCC/LPS and IS/vehicle) was measured. Data are presented as the mean + sem; $N = 8$ per experimental group; RFU = relative fluorescence units; RLU = relative luminescence units; HCC = home cage control; IS = inescapable shock; LPS = lipopolysaccharide.

Table 1.

Primer Specifications.

Gene	Primer Sequence 5' → 3'	Description
AIM2	F: AGTCCAGAAGATAACAGAGC R: TCTCCTTCCTCGCATTTTGT	Inflammasome component mediating caspase-1/IL-1 β activation
ASC	F: ACCCATAGACCTCACTGAT R: ACAGCTCCAGACTCTTCCAT	NLRP3 inflammasome adaptor
β -Actin	F: TTCCTTCCTGGGTATGGAAT R: GAGGAGCAATGATCTTGATC	Cytoskeletal protein (Housekeeping gene)
Caspase-1	F: ATGCCGTGGAGAGAAACAAG R: CCAGGACACATTATCTGGTG	Inflammasome effector mediating processing of proIL-1 β into mature IL-1 β
CD163	F: GTAGTAGTCATTCAACCCTCAC R: CGGCTTACAGTTTCTCAAG	Hemoglobin receptor expressed by macrophages, but not microglia
GFAP	F: AGATCCGAGAAACCAGCCTG R: CCTTAATGACCTCGCCATCC	Astrocyte antigen
HMGB1	F: GAGGTGGAAGACCATGTCTG R: AAGAAGAAGGCCGAAGGAGG	Damage-associate molecular pattern
IL-1 β	F: CCTGTGCAAGTGTCTGAAG R: GGGCTTGGAAGCAATCCTTA	Pro-inflammatory cytokine
IL-6	F: AGAAAAGAGTTGTGCAATGGCA R: GGCAAATTTCTGGTTATATCC	Pro-inflammatory cytokine
NF- κ B α	F: CACCAACTACAACGGCCACA R: GCTCCTGAGCGTTGACATCA	Induced by NF κ B to inhibit NF κ B function
NLRC4	F: GGACTTAACTGGACACAGTC R: CACCCAGAGGAAAGAAGTTC	Inflammasome component mediating caspase-1/IL-1 β activation
NLRP1	F: TTCGGAAGGCCATTGATGAG R: TGGAGCTGGACACGATATAG	Inflammasome component mediating caspase-1/IL-1 β activation
NLRP3	F: AGAAGCTGGGGTTGGTGAATT R: GTTGTCTAACTCCAGCATCTG	Inflammasome component mediating caspase-1/IL-1 β activation
RAGE	F: AGGACAAGCTGCAGGCTCTG R: TTCTGGGGCCTTCCTCTCCT	Receptor for HMGB1 that mediates chemotaxis and inflammatory responses.
TLR4	F: TCCCTGCATAGAGGTACTTC R: CACACCTGGATAAATCCAGC	Receptor for LPS and DAMPs
TNF	F: CAAGGAGGAGAAGTTCCTCA R: TTGGTGGTTTGCTACGACG	Pro-inflammatory cytokine