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Research report

Effects of LPS-induced immune activation prior to trauma exposure on PTSD-like symptoms in mice

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HIGHLIGHTS

- Predator stress decreased pro-/anti-inflammatory balance in the brain in mice.
- Acute LPS before stress was not sufficient to alter stress-induced behaviors.
- Acute LPS is not sufficient to increase susceptibility for PTSD-like behaviors.

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ABSTRACT

The prevalence of posttraumatic stress disorder (PTSD) is high in the armed services, with a rate up to 20%. Multiple studies have associated markers of inflammatory signaling prior to trauma with increased risk of PTSD, suggesting a potential role of the immune system in the development of this psychiatric disorder. One question that arises is if "priming" the immune system before acute trauma alters the stress response and increases enduring effects of trauma. We investigated the time course of inflammatory response to predator stress, a robust stressor that induces enduring PTSD-like behaviors, and the modulation of these effects *via* prior immune activation with the bacterial endotoxin, lipopolysaccharide (LPS), a Toll-like receptor 4 (TLR4) agonist. Mice exposed to predator stress exhibited decreased pro-/anti-inflammatory balance in the brain 6 h after stress, suggesting that predator exposure acutely suppressed the immune system by increasing anti-inflammatory cytokines levels. Acute immune activation with LPS before a single predator stress did not alter the enduring avoidance behavior in stressed mice. Our findings suggest that acute inflammation, at least *via* TLR4 activation, is not sufficient to increase susceptibility for PTSD-like behaviors in this model. Future studies will examine if chronic inflammation is required to induce similar immune changes to those observed in PTSD patients in this model.

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1. Introduction

Posttraumatic stress disorder (PTSD) is a severe and complex psychiatric disorder that affects importantly the quality of life of patients and their families. The prevalence of PTSD in the American general population is 8%, but the prevalence in the armed services is markedly increased with a rate up to 20% [1]. In the past few years, evidence for a role of the immune system in the development of PTSD symptoms has emerged [2,3]. Increased levels of the pro-inflammatory cytokines interleukin-1β (IL-1β), interleukin-2 (IL-2), interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) levels are observed in PTSD patients [1,3,4]. Furthermore, PTSD patients exhibit increased risk for several immune/inflammatory diseases, including cardiovascular disease, diabetes, and metabolic syndrome, which are observed in up to 80% of patients [5], supporting a potential role of inflammation in the pathophysiology of PTSD.

Multiple studies have associated immune dysfunction prior to trauma with increased risk for PTSD. Marines diagnosed with PTSD after deployment had two-fold higher levels of the inflammatory marker C-reactive protein (CRP) *before* deployment, indicating that Marines with high CRP levels before trauma were at higher

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risk for developing PTSD symptoms after trauma [6]. Altered gene expression in innate immune genes were also found in Marines [7] and mutations in CRP gene have been positively correlated with PTSD symptoms [8]. These findings indicated that abnormalities in inflammatory response may "prime" individuals to develop PTSD symptoms after trauma. Human studies can only offer correlational evidence that inflammation plays a role in risk for PTSD. It is also possible that inflammation is an "epiphenomenon" that is linked to some other core factor that increases risk, *e.g.* chronic stress effects, poor health habits, etc. Animal models are critical in identifying the causal mechanisms of immune factors underlying increased risk for PTSD.

To study neurobehavioral and biological mechanisms of PTSD *in vivo*, the predator stress model, consisting of exposing a rodent to a cat, mimics a similar severe trauma and elicits several PTSD-like characteristics. For example, predator exposure produces prolonged anxiety-like behaviors, altered morphology of fear-related brain structures such as the amygdala, and changes in expression of brain-derived neurotrophic factor (BDNF) and corticotropin-releasing factor (CRF) in several brain regions [9–12]. Thus, predator exposure meets the criteria for face and construct validity for an animal model of PTSD [13,14]. A study investigating the therapeutic effects of chronic treatment with selective serotonin reuptake inhibitors (SSRIs) has not been published yet using this stress paradigm, although it is responsive to prophylactic SSRI treatment and gene mutations that confer risk for PTSD [15,16], supporting the predictive validity of the predator stress model. To investigate the role of immune signaling after and prior to trauma exposure in the development of PTSD-like phenotype, we conducted two experiments. First, we studied the time course of inflammatory response after acute predator stress in mice. Second, by administering the Toll-like-receptor 4 (TLR4) agonist lipopolysaccharide (LPS), which induces inflammatory markers (*e.g.* pro-inflammatory cytokines and CRP) in blood and brain [17,18], we studied the effect of immune activation prior to acute trauma on predator stress-induced avoidance behaviors.

2. Materials and methods

2.1. Animals

1.1.1 Adult male C57BL/6 mice ($n = 108$) were used at 8–10 weeks of age (The Jackson Laboratories, CA, USA) and housed with water and food *ad libitum* and in a 12 h/12 h reverse light/dark cycle (lights on at 7:00 P.M. and off at 7:00 A.M.) before being single housed one week before predator stress exposure. For the predator stress exposure, one male cat (Liberty Research, NY, USA) was used and housed in a room with another male cat with access to food, water and enrichment devices *ad libitum*. All testing was conducted in accordance with the *Principles of Laboratory Animal Care*, National Institutes of Health guidelines, as approved by the University of California San Diego.

2.2. Predator stress exposure

One week prior to predator exposure, all mice were handled for 1 min on 5 consecutive days for habituation. On the day of testing the mouse was placed into a room ($2.3 \times 1.8\ m^2$; 150–200 lx) and exposed to a laboratory cat for 10 min [9] while cat/mouse interactions were scored. The following cat behaviors were considered as interaction with the mouse: sniffing (bringing its nose near the mouse to explore the mouse's scent), pawing (playing gently with the mouse by using its paws), and mouthing (touches the mouse with its mouth without biting). No difference of interaction time was found between saline-injected and LPS-injected mice (Supplemental Fig. 1). Control animals were exposed to handling for 1 min. No injury or death was observed during the experiments. Cats were exposed to no more that 6 mice per day to prevent habituation.

2.3. Experiment 1: inflammatory response following predator stress exposure

2.3.1. Animal groups and sample collection

Mice were assigned to control (n = 12) or predator stress (PS) groups ($n = 36$; $n = 12$ per time point). At several time points (6 h, 24 h and 7 days) after predator stress exposure, mice were sacrificed at 4:00 P.M., when inflammatory markers in mice reach their circadian peak [19]. Under isoflurane anesthesia, mice were sacrificed and brain tissues and trunk blood were collected. Brains were hemisected prior to storage. Blood was centrifuged for 20 min at 3000 rpm and 4 °C, and plasma was collected. Brain tissue and plasma samples were kept at −80 °C until analyzed for inflammatory markers. Control mice were euthanized 24 h after handling and this group was used as a control for all time points.

2.3.2. Cytokine quantification

The right hemispheres of the brain were homogenized in RIPA buffer containing protease inhibitors cocktail (Life Technologies, CA, USA). Samples were centrifuged for 15 min at 15,000 rpm and 4 °C, and supernatants were collected. Protein samples were tested for pro-inflammatory cytokines IL-1β, IL-6, TNF-α and CRP and anti-inflammatory cytokines IL-1 receptor antagonist (IL-1Ra) and IL-10 using ELISA duoset kits (R&D systems, MN, USA) following manufacturer's instructions. For plasma samples, only CRP levels were measured, since undetectable levels were found for the other inflammatory markers.

2.3.3. Statistical analysis

One-way analysis of variance (ANOVA) was used to determine the effect of time point for each inflammatory parameter. A P value <0.05 was considered significant. When appropriate, Tukey's *post hoc* comparisons were conducted following one-way ANOVA using GraphPad (GraphPad Software Inc., CA, USA).

2.4. Experiment 2: effects of immune activation prior to predator stress exposure

2.4.1. Animal groups and conditions

Mice were assigned to one of four groups (n = 15 per group): (1) saline/handled control; (2) saline/PS; (3) LPS/handled control or (4) LPS/PS. Two hours before predator stress exposure, saline or LPS (Escherichia coli 0111:B4; Sigma Aldrich, MO, USA) 0.1 mg/kg was administered intraperitoneally (i.p.). The LPS dose and time point was chosen based on data supporting the pro-inflammatory effects of this dose with this batch and serotype (Supplemental Fig. 2) and previous reports showing increased pro-inflammatory cytokines in both plasma and centrally after acute LPS [20–23]. Predator stress exposure was performed as described above. To confirm the effect of LPS, mice were weighed 24 h after injection to measure weight loss as an index of sickness behavior and a second set of mice were sacrificed 2 h after LPS injection to confirm cytokine activation at this dose and time point (Supplemental Fig. 2).

2.4.2. Behavioral testing

Post-"trauma" avoidance behaviors were assessed with three tests: (1) open field test; (2) light-dark box test; and (3) a trauma-reminder test (according to our previously published methods [15]). For all behavioral testing, mice were habituated to the room for 60 min. Seven days after predator exposure, the open field test was performed by placing the mouse in an arena ($40 \times 40 \times 40\ cm^3$; 800 lx) in which its locomotor activity was tracked for 10 min using