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Central Neuroimmune Activity and Depressive-Like Behavior in Response to Repeated Maternal Separation and Injection of LPS

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Abstract

Disruption of attachment relations in early life is linked to greater vulnerability to depressive illness at later ages. Evidence suggests this process involves stress-induced activation of central inflammatory factors, though the specific mediators and processes involved are not known. We used a guinea pig model in which effects of maternal separation appear more clearly due to absence of the attachment figure than is the case for other laboratory rodents. Separation in a novel environment on two consecutive days evoked a depressive-like behavioral response that sensitized during a final test 9 days later. At this time, prior separation blunted the response of prostaglandin synthesizing enzymes (COX-2 and mPGES) and chemokines (CXCL-1 and MCP-1) 120 min following injection with lipopolysaccharide and isolation in a novel cage. The blunted response was not associated with a greater plasma cortisol elevation. In addition, injection of saline just prior to isolation at the oldest age elicited small, but significant, elevations in several signaling molecules, particularly at 30 min. These results demonstrate lasting central inflammatory consequences of our separation procedure. However, contrary to expectations, sensitization of depressive-like behavior was not associated with an increase in expression of neuroimmune mediators to inflammatory challenge. Together with earlier findings, the results suggest a multi-step process in which inflammatory response to an initial separation affects downstream mediators to sensitize depressive-like behavior.

Keywords

maternal separation; attachment disruption; depression; depressive-like behavior; inflammation; chemokines; prostaglandins

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Declarations of Interest

None

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1. Introduction

Disruption of attachment and other forms of early social stress are critical risk factors for Major Depressive Disorder (MDD; Norman et al., 2012; Slavich & Irwin, 2014). It has been proposed that social separation, neglect, abuse, and other stressful early events sensitize stress-responsive neural and/or neurochemical mechanisms [e.g., amygdala activity, corticotropin-releasing factor release] so that, in vulnerable individuals, stressors in later life elicit disproportionate and uncontrolled reactions that constitute, or transition into, the depressive episode (Gillespie & Nemeroff, 2007; Gold et al., 1988). Recent studies strongly implicate inflammatory mechanisms in this process (Slavich & Irwin, 2014). Early-life stress can upregulate inflammatory activity in later life (Gouin et al., 2012; Slopen et al., 2015), and increased inflammatory activity promotes development of MDD (Dantzer et al., 2011; Raedler, 2011). Further, inflammation appears preferentially associated with depression occurring following early-life stress (Danese et al., 2008; Miller & Cole, 2012). Studies in humans have primarily relied on peripheral inflammatory responses as proxies for central changes, though inflammatory markers in cerebrospinal fluid and postmortem brain support the involvement of CNS inflammation (Raedler, 2011).

In rats, maternal separation or other disturbances that alter maternal behavior produce various alterations in central measures of inflammation (Bodnar et al., 2017; Brenhouse & Andersen, 2011; Roque et al., 2016; Saavedra et al., 2017). These outcomes appear largely due to altered maternal care (e.g., Liu et al., 1997; Walker et al., 2017) or withdrawal of specific forms of maternal stimulation that regulate the physiology and behavior of the altricial young (Hofer, 1987; Levine, 2002). While valuable, these studies provide little information about the consequences of removal of the attachment figure per se. In contrast to rats, guinea pig pups are born physically mature (Blatteis, 1975; Harper, 1976; Schiml & Hennessy, 1990) and display evidence of selective attachment to the mother (Hennessy & Ritchey, 1987; Jäckel & Trillmich, 2003; Pettijohn, 1979). Maternal care, in turn, is minimal. Lactating guinea pigs do not build nests and exhibit negligible active maternal care after the first few days of life (Hennessy & Jenkins, 1994; König, 1985). Therefore, effects of maternal separation can be attributed to absence of the attachment figure, rather than deprivation of maternal care or altered maternal care upon reunion with the mother. Further, because the pups are physically mature it is unlikely that outcomes of separation result from removal of physiological regulation provided by maternal stimulation (Hofer, 1967; Levine, 2002).

Isolation of guinea pig pups in a brightly lit novel environment elicits an initial active behavioral stage of vocalizing and locomotor activity followed by a second passive stage characterized by an immobile crouched stance, prolonged eye-closure, extensive piloerection, and apparent disinterest in the environment (Hennessy et al., 1995). These two stages are reminiscent of the “protest” followed by “despair” or “anacletic depression” originally reported in maternally separated human (Spitz, 1946) and nonhuman (Mineka & Suomi, 1978) primate infants, though in the guinea pig the full response unfolds in a matter of hours, rather than days or weeks. Absence of the maternal attachment figure is crucial since the mother’s presence in the novel environment greatly suppresses the depressive-like second stage, whereas as the presence of other conspecifics has limited or no effect

(Hennessy & Morris, 2005; Hennessy et al., 2015). Three lines of evidence indicate that the second stage is mediated by inflammatory activity. First, direct inflammatory challenge with lipopolysaccharide (LPS) elicits the same constellation of passive behaviors (Hennessy et al., 2004). Second, the separation procedure produces physiological indicators of an inflammatory response, specifically, fever (Hennessy et al., 2010) and increased expression of the proinflammatory cytokine TNF- α in spleen (Hennessy et al., 2007a). Third, depressive-like behaviors during separation can be reduced with administration of anti-inflammatory agents (e.g., Hennessy et al., 2007b; Perkeybile et al., 2009).

Furthermore, as is hypothesized to occur when MDD develops following early-life stress, the passive response sensitizes with repeated separation so that increased time is spent exhibiting depressive-like behavior during a second separation the next day as well as a third separation 9 days after the second (Schneider et al., 2012). The fever response also sensitizes with a greater and more-rapidly developing fever during subsequent separations (Hennessy et al., 2010; Schneider et al., 2012). The sensitization, like the initial response, can be reduced with administration of anti-inflammatory agents (Hennessy et al., 2011; 2015). Together, these findings indicate that a several-hour period of separation of guinea pig pups from the attachment figure in a threatening (i.e., novel, brightly lit) environment evokes an inflammatory-mediated depressive-like response that sensitizes with repeated separation. The validity of this model for the study of depression is supported by the recent finding that an initial 3-hour separation also increased later depressive-like responding in the most widely used screen for antidepressant medication, i.e., previous separation increased immobility in the forced swim test (Hennessy et al., 2017).

Despite peripheral signs of inflammatory activity (e.g., fever) we have yet to detect central inflammatory responses. In a series of unpublished experiments, separation produced no change in expression or levels of various inflammatory signaling molecules in discrete brain regions. These findings may reflect insufficient sensitivity of the procedures, or inappropriate choice of time points, brain regions, or target molecules. In the current study, we adopted a different approach. We know from early pilot work (Hennessy et al., 2004) that injecting a guinea pig pup with LPS and then exposing it to a novel environment elicits a robust IL-1 β response in hypothalamus, an area of particular interest because of its role in control of fever (Netea et al., 2000). We reasoned that if early separation sensitized the later inflammatory response, and LPS increased the response to a measurable level, the influence of earlier separation might be apparent in hypothalamus. Therefore, we examined whether previous separation would increase hypothalamic expression of neuroimmune signaling molecules in responses to LPS. In addition to IL-1 β , we included two other commonly studied proinflammatory cytokines, TNF- α and IL-6. We also assessed expression of the chemokines CXCL-1 and MCP-1, which have been shown to exhibit sensitized responses to LPS in rat hypothalamus following repeated cold exposure (Girotti et al., 2011). Finally, the panel included mPGES, a prostaglandin (PGE-2) synthase, and cyclooxygenase (COX) 1 and 2. We were particularly interested in the latter three molecules because COX inhibitors reduced the depressive-like response, fever, and their sensitization, suggesting mediation by PGE-2 (Hennessy et al., 2007b; Hennessy et al., 2015). Because differences in levels of glucocorticoids, which suppress inflammatory activity (Munck et al., 1984), could account for differences in gene expression, we also assessed levels of plasma cortisol, the primary

glucocorticoid of the guinea pig (Dalle & Delost, 1974). Our first experiment was conducted to choose a dose of LPS having a submaximal effect on expression of inflammatory molecules so that further increases might be detected. In the next three experiments, ~ 3-week-old pups were either separated or not on two consecutive days, and then 9 days after the second separation, injected with either LPS or saline and isolated until tissue collection. Undisturbed controls were included in each experiment. These experiments differed in the time point at which tissue was harvested—either 30, 60, or 120 min following injection.

2. Materials and methods

2.1 Subjects

Albino Hartley guinea pigs (*Cavia porcellus*) were bred and housed in our laboratory. The parents had been purchased from Hilltop Lab Animal Inc, Scottsdale PA. Following birth (Day 0) mother and litter were maintained in an opaque plastic cage (73 × 54 × 24 cm) with a wire front and sawdust bedding. Food and water were available *ad libitum*. The colony room was kept at ~70°F on a 12:12 light/dark schedule with lights on at 0700 hr. Pups were housed continuously with the mother with the exception of behavioral testing to be described below. All procedures were in compliance with Public Health Service guidelines and were approved by the Wright State University Institutional Animal Care and Use Committee.

2.2 Experimental conditions and test procedure

In Experiment 1, four guinea pigs (2 males, 2 females), were assigned to each of six conditions. In these conditions, pups were either undisturbed prior to brain collection or received intraperitoneal injections of either 0, 25, 50, 100, or 250 µg/kg body weight of LPS (Lot #8274, Sigma Corp.) in sterile saline vehicle just prior to isolation for 120 min and harvesting of the brain. Because this was a preliminary experiment intended solely to determine dose sensitivity to LPS, minimal samples sizes were used. To control for litter effects, no more than one pup from a litter was assigned to any one condition.

Experiments 2–4 each had five conditions: Undisturbed, Non-Separated Vehicle, Separated Vehicle, Non-Separated LPS, and Separated LPS, with 9–11 guinea pigs (4–6 males, 4–7 females) assigned to each condition. In the Undisturbed condition, offspring were left in the home cage with mother and any siblings until brain collection. In the Non-Separated Vehicle and Non-Separated LPS conditions, animals remained in the home cage until the test day (same age as for previously separated animals), when they were injected with either the saline vehicle or LPS and immediately isolated in a novel environment prior to brain collection. In the Separated Vehicle and Separated LPS conditions, pups were isolated twice for 3 hours at a 24-hour inter-separation interval between Days 19 and 22 (weaning occurs at ~Day 25; Schiml & Hennessy, 1990). Then 9 days after the second separation, animals were injected with either vehicle or LPS just prior to isolation in a novel environment and tissue collection. These ages are at about the midpoint of 20-day range at which separations have been shown to sensitize depressive-like behavior and fever (Schneider et al., 2012). We first isolated animals for 120 min as in Experiment 1. Based on these results, and earlier findings that sensitization can manifest as an earlier as well as greater response (Johnson et al., 2002;

Schneider et al., 2012), we did additional experiments in which pups were isolated for either 30 or 60 min to examine earlier time points following injection. For ease of presentation, however, we present these experiments here in temporal order, i.e., 30, 60, and 120 min of isolation, as Experiments 2, 3, and 4, respectively. However, values for a single signaling molecule are shown in the same figure for ready comparison across time points. No more than one pup from a litter was tested in any one condition with five exceptions. In these cases (one or two in each experiment) two littermates were tested in the Undisturbed condition.

All isolations (both the preweaning separations in Experiments 2–4, and the isolation following injection in all experiments) occurred in a novel, brightly lit, clear plastic cage (47 × 24 × 20 cm) in an observation suite adjacent to the room in which animals were injected. Brains were collected between 1700 and 1900 hours in Experiment 1, and between 1315 and 1700 hours in Experiment 2–4 and occurred between Days 29–31 in all experiments.

2.3 Behavioral observations

We observed behavior during Experiment 4, in which the isolation period following injection on the test day was long enough (120 min) for appreciable passive, depressive-like behavior to occur. The observation suite consisted of two rooms: one in which the animal in the test cage was positioned in front of a one-way glass window, and a second room from which the observer (85% inter-observer reliability) viewed the animal through the one-way glass. Observations occurred during Min 0–30, 60–90, and 150–180 of the initial preweaning separations, and during Min 0–30 and 90–120 of the test day. Our measure of passive, depressive-like behavior was the “full passive” response (Hennessy et al., 2007b), defined as the number of 1-min intervals in which the subject engaged in all three individual passive behaviors characteristic of the second stage of separation: (1) a distinctive crouched stance with feet pulled close to body—sometimes transitioning into lying on the cage floor with the trunk supporting the body; (2) complete or near complete closure of one or both eyes for greater than 1 second; (3) piloerection over more than half of the body.

2.4 Neural tissue collection and processing

Animals were quickly decapitated following removal from the home cage (Undisturbed condition) or following testing. Brains were then rapidly removed, immediately flash frozen in isopentane, and stored at -80° . At the time of assay, brains were briefly thawed and hypothalami were micro-dissected for subsequent extraction of RNA, cDNA synthesis, and real time RT-PCR as described recently (Doremus-Fitzwater et al, 2018). Briefly, tissue was homogenized in a Tissue Lyser (Qiagen, Valencia, CA) and RNA extractions were performed using Trizol® RNA reagent (Invitrogen, Grand Island, NY). Samples were then purified through RNeasy mini columns (Qiagen, Valencia, CA). RNA yield and quality was determined using a Nanodrop micro-volume spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, DE). Synthesis of cDNA was performed on 0.1–1.0 µg of normalized total RNA from each sample using the QuantiTect® Reverse Transcription Kit (Cat. No. 205313, Qiagen, Valencia, CA) which included a DNase treatment step.

2.5 Reverse-transcription polymerase chain reaction (RT-PCR)

Probed cDNA amplification was performed using IQ SYBR Green Supermix (Bio-Rad, cat. no. 170–8882, Hercules, CA), run in triplicate in a 384 well plate (BioRad, cat. no. HSP3805) and captured in real-time using a PCR detection system (BioRad, model no. CFX384). Specificity of reactions were determined in part through a melt curve analysis, with a single peak expressed as the negative first derivative of the change in fluorescence as a function of temperature indicating primer specificity to the target gene. β -actin was used as a reference gene. Primer sequences can be found in Table 1. Data were expressed a per cent of control using the $2^{-(\Delta\Delta C_T)}$ method (Livak & Schmittgen, 2001).

2.6 Blood collection and cortisol assay

Trunk blood was collected on EDTA following rapid decapitation with plasma separated by centrifugation and frozen until assay with commercially available enzyme-linked immunosorbent assay (ELISA) kit (Enzo Life Sciences, Farmingdale, NY). Inter-assay variability was 2.3%.

2.7. Data analysis

In Experiment 1, gene expression of injected animals was assessed by one-way (Dose) analysis of variance (ANOVA). For this preliminary study with minimal sample sizes, we used the relatively less-conservative Fisher LSD test for post-hoc paired-comparisons of significant effects. In Experiments 2–4, gene expression and cortisol were analyzed by factorial ANOVAs with Injection (saline or LPS) and Experience (previous separation or no separation) as factors. Significant interactions were examined further with tests for simple main effects (Winer, 1971). For gene expression and cortisol, Undisturbed animals were compared to Non-Separated Vehicle animals with *t*-tests for independent samples. Behavior during the first vs the second separation for the Separated Vehicle and Separated LPS groups (which had been treated identically up to that point) was compared with a dependent samples *t*-test. Behavior of animals of all four experimental groups on the final day was assessed by 2 (Injection) x 2 (Experience) ANOVA. When significant main effects were qualified by a significant interaction, only results for the interaction are reported. Although we ensured that both males and females were represented in the data, sex was not included in ANOVAs for gene expression and behavior because of small and somewhat uneven sample sizes of animals of each sex and a consistent lack of sex differences in passive behavior during separation in past studies (e.g., Hennessy et al., 2007b; 2010; 2011; 2015; Perkeybile et al. 2009). For plasma cortisol, for which sex differences among young guinea pigs have commonly been observed (Hennessy & Moorman, 1989; Hennessy & Ritchey, 1987), we confirmed that there were no significant interactions of Sex with the variables of interest (Injection, Experience), and sex was then not considered further. A probability value of 0.05 (2-tailed) was taken as indication of statistical significance. In some cases, individual group sizes for gene expression were reduced by one due to loss of sample. Because some plasma samples were lost, or at other times were available when the animal's hypothalamus was not, sample sizes for cortisol ranged from 9–12.

3.0 Results

3.1 Experiment 1. Dose response.

Significant effects of Dose were obtained for IL-1 β , $F(4, 15) = 3.64$, $p < 0.05$, COX-2, $F(4, 15) = 13.33$, $p < 0.001$, mPGES, $F(4, 15) = 8.75$, $p < 0.001$, and MCP-1, $F(4, 15) = 3.66$, $p < 0.05$. These effects reflected increasing gene expression with higher doses of LPS, as indicated by significant paired-comparisons illustrated in Figure 1. Based on these results, we chose the 25 $\mu\text{g/kg}$ body weight dose for use in Experiments 2–4. This dose appeared to be at about threshold for activating gene expression of the signaling molecules, and so would allow further increases to be observed.

3.2 Experiment 2. Effects of prior separation and LPS 30 min after injection.

3.2.1. Neuroimmune gene expression—There were numerically small, but statistically significant, differences between the Undisturbed and Non-Separated Vehicle groups in expression of IL-1 β , $t(19) = 2.23$, $p < 0.05$, CXCL-1, $t(19) = 3.01$, $p < 0.01$, and MCP-1, $t(19) = 2.34$, $p < 0.05$ (Fig. 2 & 3, 1st column). That is, a saline injection together with 30 min of isolation elevated cytokine and chemokine expression in post-weaning guinea pigs. Injection of LPS elevated expression of several signaling molecules at 30 min, as indicated by significant main effects of Injection for COX-1, $F(1, 37) = 6.22$, $p < 0.05$, COX-2, $F(1, 37) = 12.84$, $p < 0.001$, CXCL-1 $F(1, 37) = 6.02$, $p < 0.001$, and MCP-1, $F(1, 37) = 15.54$, $p < 0.001$. In no instance, however, was there a main or interactive effect implicating prior separation experience.

3.2.2. Plasma cortisol—Injection of saline followed by 30 min of isolation significantly elevated plasma cortisol levels relative to Undisturbed controls, $t(19) = 5.45$, $p < 0.001$ (Fig. 4, 1st column). Further, ANOVA yielded a significant interaction of Injection $\times$ Experience, $F(1, 33) = 5.61$, $p < 0.05$. Post-hoc tests revealed that animals that had been separated earlier and then were injected with saline had higher plasma cortisol levels at 30 min than did those that had been separated and injected with LPS, $p < 0.05$, or those that had not previously been separated and were injected with saline, $p < 0.05$.

3.3 Experiment 3. Effects of prior separation and LPS 60 min after injection.

3.3.1. Neuroimmune gene expression—There were no statistical differences in gene expression between the Undisturbed control and the Non-Separated Vehicle groups 60 min following vehicle injection, though in light of the results at 30 min, it is of interest that expression of three molecules, IL- β , IL-6, and CXCL-1 tended to again be increased by saline injection followed by isolation, p 's of 0.055 to 0.073 (Fig. 2 & 3, 2nd column; also note differences in scale for ordinate for some molecules at different time points for Fig. 3). LPS significantly elevated expression of COX-2, $F(1, 34) = 4.84$, $p < 0.05$, mPGES, $F(1, 34) = 4.79$, $p < 0.05$, CXCL-1, $F(1, 34) = 9.56$, $p < 0.005$, and MCP-1, $F(1, 34) = 6.98$, $p < 0.05$, as indicated by main effects of Injection. There also was a main effect of Experience for IL-1 β , $F(1, 34) = 4.18$, $p < 0.05$, with lower expression of IL-1 β in previously separated animals, $p < 0.05$. In addition to these main effects, there was a significant interaction of Injection $\times$ Experience for IL-6, $F(1, 34) = 4.44$, $p < 0.05$, with LPS modestly reducing IL-6 expression in previously separated guinea pigs, $p < 0.05$.

3.3.2. Plasma cortisol—At 60 min, the Non-Separated Vehicle group again had higher cortisol levels than did Undisturbed controls, $t = 3.44$, $p < 0.005$ (Fig. 4, 2nd column). Cortisol concentrations in all groups isolated for 60 min were comparable as shown by lack of main or interactive effects of Injection and Experience. Notably the higher cortisol levels of pups in the Separated Vehicle group at 30 but not 60 min suggests this group showed a faster onset or rise time of plasma cortisol (i.e., quicker reaction to novelty when not impaired by LPS) rather than a greater maximum response.

3.4 Experiment 4. Effects of prior separation and LPS 120 min after injection.

3.4.1. Neuroimmune gene expression.—There were differences in gene expression between the Undisturbed and Non-Separated Vehicle groups, with injection followed by 120 min of isolation elevating expression of COX-2, $t(18) = 2.23$, $p < 0.05$, but reducing expression of IL-1 β , $t(18) = 3.35$, $p < 0.005$, and COX-1, $t(18) = 2.62$, $p < 0.05$ (Fig. 2 & 3, 3rd column). Main effects of Injection were observed for IL-1 β , $F(1, 35) = 13.12$, $p < 0.001$, and IL-6, $F(1, 35) = 5.57$, $p < 0.05$ with expression of IL1 β increased, and IL-6 reduced, 120 min following LPS injection as compared to following injection of saline. There was a significant interaction of Injection x Experience for COX-1, $F(1, 35) = 5.09$, $p < 0.05$, but post hoc tests revealed no differences between pairs of means. Of most interest, however, were significant interactions for COX-2, $F(1, 35) = 5.56$, $p < 0.05$, mPGES, $F(1, 35) = 9.48$, $p < 0.005$, CXCL-1, $F(1, 35) = 6.86$, $p < 0.05$, and MCP-1, $F(1, 35) = 5.65$, $p < 0.05$ (Fig. 3). In each case, LPS produced a marked elevation in expression relative to saline in both previously separated and nonseparated guinea pigs, p 's < 0.01 , but the increase was significantly blunted in those that had been separated on two occasions during the preweaning period, all p 's < 0.01 .

3.4.2. Plasma cortisol—Injection of saline in previously non-separated pups again elevated plasma cortisol levels relative to Undisturbed controls, $t(18) = 5.83$, $p < 0.001$ (Fig. 4, 3rd column). LPS injection clearly increased cortisol levels at 120 min, $F(1, 31) = 26.24$, $p < 0.001$. Yet, unlike the case for gene expression of several signaling molecules, the effect of LPS did not vary with separation history, as shown by lack of a significant interaction.

3.4.3. Behavior—In Experiment 4, 12 of 20 pups separated during the preweaning period exhibited the full passive response during more 1-min intervals in the second as opposed to the first separation, but there was no difference in mean levels (Separation 1: $M = 34.25 \pm 2.87$; Separation 2: $M = 35.70 \pm 3.07$). By the test day, however, sensitization of the full passive response was evident, as indicated by a main effect of Experience, $F(1, 36) = 5.38$, $p < 0.05$ in the 2 (Injection) x 2 (Experience) ANOVA (Table 2). That is, as in numerous previous experiments with this paradigm, the passive depressive-like response sensitized with repeated separation (e.g., Hennessy et al., 2011, 2015; Schneider, 2012), though in this case only during the third separation. The main effect of Injection approached significance, $F(1, 36) = 3.21$, $p < 0.09$, with the 25 $\mu\text{g/kg}$ dose of LPS tending to increase passive behavior during the 2-hour period of isolation.

4.0 Discussion

The means by which disruption of filial attachment can promote depression has been a focal point for developmental and psychiatric research for many decades (Bowlby, 1952; Spitz, 1946). While increasing evidence points to inflammatory mechanisms as mediators of long-term vulnerability to MDD (Slavich & Irwin, 2014), precise central mechanisms have remained elusive. Studies with rats and mice have demonstrated lasting inflammatory effects of perturbation of early mother-infant interactions (Bodnar et al., 2017; Brenhouse & Andersen, 2011; Roque et al., 2016; Saavedra et al., 2017), but these effects are not necessarily the result of attachment disruption, but rather to alterations in maternal care, as discussed above. The strong evidence for attachment in guinea pig pups, together with the finding of persistent elevations of depressive-like behavior following maternal separation (e.g., Schneider et al., 2012), suggest studies in this species may be able to provide useful insights into central inflammatory processes that are involved. Several findings of the current study offer potential clues.

First, attachment disruption did not augment the central inflammatory response to a modest dose of LPS, as both predicted here and previously observed following various forms of early postnatal or prenatal stress (Diz-Chaves et al., 2013; Ehrlich et al., 2016; Levine et al., 2015; Réus et al., 2017; Roque et al., 2016). Rather, earlier separation that was sufficient to increase depressive-like behavior at the oldest age blunted the expression of mPGES and COX-2, two enzymes responsible for PGE-2 synthesis, as well as of CXCL-1 and MCP-1, two chemokines previously shown to increase expression with repeated stress exposure (Girotti et al., 2011; Sawicki et al., 2015). In the guinea pig, sensitization of the depressive-like passive response is accompanied by sensitization of fever (Hennessy et al., 2010; Schneider et al., 2012). Because fever is mediated by PGE-2 in hypothalamus (Netea et al., 2000), and because inhibitors of prostaglandin synthesis can reduce the passive behavior and fever in guinea pigs, it is particularly interesting that LPS reduced expression of two enzymes underlying PGE-2 synthesis in hypothalamus at 120 min, a time point intended to capture the peak of the neuroimmune response.

In light of evidence that prior stress may accelerate as well amplify inflammatory responsiveness (Hennessy, et al., 2010; Johnson et al., 2002; Schneider et al., 2012), we included time points in the current study to capture possible earlier responses. Yet, we saw no sign of increased expression of any signaling molecule to LPS at 30 or 60 min in previously separated guinea pigs. Thus, the blunting seen at 120 min appears to represent a true reduction in expression of neuroimmune molecules in response to LPS, rather than a leftward shift in the time course of response. While this blunting of expression was not predicted, it also is not without precedent. Early-life social separation was found to reduce later peripheral inflammatory response to LPS in mice, rats, and piglets (Avitsur et al., 2013; Roque et al., 2016; Saavedra et al., 2018; Tuchscherer et al., 2004), and later inflammatory pain sensitivity in rats (Genty et al., 2018). As for the CNS, prenatal exposure to alcohol reduced hypothalamic levels of IL-1 β , TNF- α , and IL-6 in preweaning rats (Bodnar, 2017), and earlier separation reduced the TNF- α response, though increased the IL-1 β response, to LPS in the hippocampus of piglets (Tuchscherer et al., 2004). Finally, it does not appear the blunted response can be attributed to greater suppressive action of cortisol because the final

separation test and injection of LPS produced comparable elevations of plasma cortisol concentrations in previously separated and non-separated guinea pigs.

A second aspect of the current findings that may be of translational relevance is the response of chemokines. Studies of inflammatory-related depression or depressive-like behavior have typically focused on classic proinflammatory cytokines (e.g., IL-1 β , IL-6, TNF- α). However, there is increasing evidence for involvement of chemokines, including those measured here, in mental disorders including depression (Lehto et al., 2010; Merendino et al., 2004; Stuart & Baune, 2014). Central expression of both CXCL-1 and MCP-1 have also been found to be increased following repeated social defeat in mice (Sawicki et al., 2015). In the present study, CXCL-1 and MCP-1 expression in response to LPS was clearly blunted by earlier social stress, whereas only minimal, nonsignificant reductions in classic cytokines were observed. Together these results suggest studies of early-life stress on vulnerability for developing depressive illness might profit from greater attention to altered chemokine activity.

Third, it is of interest that an initial period of separation following saline injection produced small, but significant, elevations in expression of signaling molecules, particularly at the 30 min time point. These increases are our first examples of a psychogenic stressor increasing central proinflammatory activity in infant guinea pigs. Detection here might have been due to the choice of time point or improvements in primer specificity for guinea pigs, as gene sequences for guinea pigs (a rather infrequently used rodent for biomedical research) have improved substantially. While quite modest compared to responses evoked by LPS, these increases potentially reflect processes underlying the initial induction of passive, depressive-like behavior in separated guinea pig pups. However, additional tests confirming such responses occur in separated preweaning pups in the absence of saline injection in our standard test situation are needed before this possibility can be meaningfully pursued.

In earlier studies reporting a reduction of cytokine responses following early-life stressors, other measures indicating enhanced inflammation also were observed (Bodnar et al., 2016; Roque et al., 2016; Saavedra et al., 2018). That is, the blunting appeared not to reflect a reduction in overall inflammatory response, but rather one element of a more complex process. The reduced neuroimmune signaling response to LPS in guinea pigs that had experienced prior separation in the present study should be interpreted in the context of findings (Hennessy et al., 2011; 2015) that treatment with anti-inflammatory agents—either naproxen or IL-10—prior to an initial separation reduced the depressive-like behavioral response to subsequent separation episodes. These data indicate that inflammatory mechanisms are involved in the initiation of the sensitization process, while the current findings suggest that the process is not simply one of greater cytokine release to later stressors. One possibility is that the number or sensitivity of chemokine receptors is increased, or that some other aspect of the downstream actions of an initial inflammatory response are enhanced, so that the reduced expression of signaling molecules observed here is a compensatory effect limiting the ultimate inflammatory response. It is also possible that an initial inflammatory response sensitizes non-inflammatory neural or neurochemical processes. Studies examining the effect of anti-inflammatory agents administered after the initial separations but before testing at 30 days of age (see Wieck et

al., 2013 for example in rats) may be useful in better determining whether there is an inflammatory component to the putative sensitized downstream process. One must also consider that the strategy employed here was to use injection of LPS followed by isolation to infer sensitization to later separation alone. Presumably LPS would amplify the response to separation itself so that differences due to previous separation history could be more-easily measured. However, we cannot rule out the possibility that LPS produced a different pattern of central cytokine response than would separation alone, such that the blunting of expression we observed in previously separated animals was specific to LPS injection, as opposed to social separation.

We purposely chose a dose of LPS that was close to threshold so that further increases due to previous separation history could be produced. In the preliminary experiment (Experiment 1), this dose clearly increased expression of some signaling molecules (COX-2, mPGES), but not others, notably the classic proinflammatory cytokines: IL-1 β , IL-6, and TNF- α . It was not unexpected then that this dose—which was lower than used in our previous studies—was insufficient to consistently elevate signaling molecule expression or plasma cortisol levels in Experiments 2–4, or that it only tended to increase passive, depressive-like behavior in Experiment 4. One curious effect of LPS was that it slightly, but significantly, reduced levels of IL-6 expression following LPS injection at 60 and 120 min. We cannot unequivocally rule out the possibility that LPS produced an initial rise that was not captured at the 30-min sample, followed by a decline to below the levels of saline-injected animals at 60 and 120 min. A pattern of this sort was seen in the response of IL-1 β to injection of saline. In the Non-Separated Vehicle group, IL-1 β expression was greater than that of the Undisturbed controls at 30 min, but lower than that of the controls at 120 min. Finally, it should be noted that while our study focused on inflammatory factors in the hypothalamus, a site known to be highly sensitive to the influence of stressors (Deak et al, 2005), and crucial for thermoregulation (Saper, 1998), it remains possible that different patterns of signaling molecule expression might have been observed in other CNS sites sensitive to the influence of attachment-figure separation.

In sum, the present results show that the separation procedure used here alters expression of central neuroimmune signaling molecules during subsequent inflammatory challenge. These effects appear due to the psychogenic stressor of attachment-figure separation in a threatening environment, rather than to a physical challenge posed by the procedure on an immature nervous system, or to altered maternal care upon return to the home cage. Together with previous findings, the results suggest a multi-step process in which an initial inflammatory response heightens the activity of a secondary mediator that underlies sensitization of depressive-like behavior.

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Highlights

- Effects of attachment-figure separation on depression modeled in guinea pigs
- Early separation increased later depressive-like response to separation
- Separation blunted later response of COX-2, mPGES, CXCL-1, and MCP-1 to LPS
- Psychogenic stress modestly increased response of IL-1 β , CXCL-1 and MCP-1 to LPS

Figure 1.

Mean gene expression of neuroimmune signaling molecules in the hypothalamus of guinea pigs either under undisturbed conditions or 120 min following injection of saline or one of four doses of LPS. Vertical lines represent standard errors of the mean. Circles indicate individual data points. Injected groups with different letters differ significantly, $p < 0.05$.

Figure 2.

Mean gene expression of IL-1 β , IL-6, TNF- α and COX-1 in the hypothalamus of guinea pigs either under Undisturbed conditions or 30, 60, or 120 min following injection of saline or 25 μ g/kg LPS in animals that had either had or had not undergone separation 9 and 10 days earlier. Vertical lines represent standard errors of the mean. Circles indicate individual data points. Significant differences ($p < 0.05$) are indicated by a star for comparisons (t -test) between Undisturbed and Separated Vehicle conditions and by an asterisk for comparisons (ANOVA) among the 4 Injection x Experience conditions.

Figure 3.

Mean gene expression of COX-2, mPGES, CXCL1, and MCP-1 in the hypothalamus of guinea pigs either under Undisturbed conditions or 30, 60, or 120 min following injection of saline or 25 µg/kg LPS in animals that had either had or had not undergone separation 9 and 10 days earlier (note differences in ordinates for different time points). Vertical lines represent standard errors of the mean. Circles indicate individual data points. Significant differences ($p < 0.05$) are indicated by a star for comparisons (t -test) between Undisturbed and Separated Vehicle conditions and by an asterisk for comparisons (ANOVA) among the 4 Injection x Experience conditions.

Figure 4.

Mean plasma cortisol levels of guinea pigs either under Undisturbed conditions or 30, 60, or 120 min following injection of saline or 25 μg/kg LPS in animals that had either had or had not undergone separation 9 and 10 days earlier. Vertical lines represent standard errors of the mean. Circles indicate individual data points. Significant differences ($p < 0.05$) are indicated by a star for comparisons (t -test) between Undisturbed and Separated Vehicle conditions and by an asterisk for comparisons (ANOVA) among the 4 Injection x Experience conditions.

Table 1.

Primers used in all experiments

Target	Accession Number	Primer Direction	Sequence
IL-1 β	NM_001172968.1	Forward primer	AAAAATGCCAGGTCTCTGAAATAGC
		Reverse primer	CAGCTTGATCCCCTCATCCA
IL-6	XM_003475371.2	Forward primer	CACATCACAGAGGTGCATACTACT
	predicted sequence	Reverse primer	TGAGGCCAATGCAGAGGAGA
TNF- α	NM_001173025.1	Forward primer	GGGAAGAGCAGTTCTCCAGTG
		Reverse primer	AGCCACCGGCTTGTCATTAT
MPGES-1	XM_003470944.2	Forward primer	GGAAGAAGGCTTTTGCCAACC
	predicted sequence	Reverse primer	TCTCCATGTCATTGCGGTGG
Cox-1	XM_003470662.2	Forward primer	GCGCCATGAGCCGGAG
	predicted sequence	Reverse primer	CGTAGTGCTCAAGGCCAAAG
Cox-2	Y07896.1	Forward primer	CTCCTGCGCAATGCAATCA
	(within NM_001173007.1)	Reverse primer	TCCTTCTTCCCTTTACACCC
MCP-1	NM_001172926.1	Forward primer	CCAACACTCCACCATGCAGA
		Reverse primer	GGGGTATTAACTCCATCCGGC
CXCL1 GROA	NM_001172938.1	Forward primer	AGAACATCCAGAGCGTAGCG
		Reverse primer	AAGTGGCTTTGCTTCCTTTCAG

Table 2.

Mean and standard error (se) of the number of 1-min intervals that previously separated and non-separated guinea pigs displayed the depressive-like full passive response following injection of saline or LPS

	Vehicle [#]	LPS
Non-Separated [*]	16.1 (3.0)	22.6 (3.3)
Separated	24.3 (4.0)	28.2 (2.6)

^{*} differs from Separated, $p < 0.05$

[#] differs from LPS, $p < 0.09$