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Effects of early-life adversity on immune function are mediated by prenatal environment: Role of prenatal alcohol exposure

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ABSTRACT

The contribution of the early postnatal environment to the pervasive effects of prenatal alcohol exposure (PAE) is poorly understood. Moreover, PAE often carries increased risk of exposure to adversity/stress during early life. Dysregulation of immune function may play a role in how pre- and/or postnatal adversity/stress alters brain development. Here, we combine two animal models to examine whether PAE differentially increases vulnerability to immune dysregulation in response to early-life adversity. PAE and control litters were exposed to either limited bedding (postnatal day [PN] 8-12) to model early-life adversity or normal bedding, and maternal behavior and pup vocalizations were recorded. Peripheral (serum) and central (amygdala) immune (cytokines and C-reactive protein – CRP) responses of PAE animals to early-life adversity were evaluated at PN12. Insufficient bedding increased negative maternal behavior in both groups. Early-life adversity increased vocalization in all animals; however, PAE pups vocalized less than controls. Early-life adversity reduced serum TNF-α, KC/GRO, and IL-10 levels in control but not PAE animals. PAE increased serum CRP, and levels were even higher in pups exposed to adversity. Finally, PAE reduced KC/GRO and increased IL-10 levels in the amygdala. Our results indicate that PAE alters immune system development and both behavioral and immune responses to early-life adversity, which could have subsequent consequences for brain development and later life health.

1. Introduction

Brain development is a dynamic and continuous process that starts very early in prenatal life and extends through adolescence (Andersen, 2003; O'Mahony et al., 2017). Exposure to adversity and/or stress in any of these life stages can negatively alter the neurodevelopmental trajectory, putting the individual on a pathway to pathology. Among many potential adverse environmental factors, alcohol, in addition to its teratogenic effects, can program developing neurobiological systems, altering brain development and increasing vulnerability to cognitive and behavioral deficits, as well as physical and mental health problems (Hellemans et al., 2010a; Riley et al., 2011; Schneider et al., 2011; Valenzuela et al., 2012; Weinberg et al., 2008). Importantly, exposure to alcohol during gestation carries with it an increased risk of being exposed to adverse and/or stressful environments during postnatal life (O'Connor and Kasari, 2000; O'Connor and Paley, 2006; Streissguth et al., 2004). Moreover, consistent findings indicate that early postnatal adversity such as neglect, abuse, and/or maltreatment – especially from the caregiver – can also change brain development and have long-lasting consequences for the physical and mental health of the offspring (Chen and Baram, 2016; Cirulli et al., 2009; Danese and McEwen, 2012; Drury et al., 2016; Heim et al., 2010; McEwen, 2008; Raineki et al., 2012; Teicher et al., 2003). Nevertheless, relatively few studies have investigated how exposure to adverse and/or stressful environments early in postnatal life contributes to the pervasive and long-lasting negative effects of PAE (Alberry and Singh, 2016; Price et al., 2017).

A leading mechanistic hypothesis about how pre- and/or postnatal adversity can affect brain development suggests that dysregulation of the normal cytokine balance may play a role (Ganguly and Brenhouse, 2015; Hennessy et al., 2010; Miller et al., 2011; Nusslock and Miller, 2016). Cytokines are potent neuromodulators of brain development, affecting neurogenesis, neuronal migration, synaptogenesis, and synaptic pruning (Bajetto et al., 2001; Bessis et al., 2005; Deverman and Patterson, 2009; Smith et al., 2007; Stephan et al., 2012). As a result, altered cytokine balance may affect many important neuronal processes, resulting in abnormal brain development and increased vulnerability to adverse adaptive, functional and health outcomes in later life (Babri et al., 2014; Bauman et al., 1997; Bilbo and Schwarz, 2009; Ganguly

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and Brenhouse, 2015; Meyer et al., 2009). Importantly, it has been shown that exposure to adverse environmental factors during sensitive early-life periods can result in a long-lasting, enhanced proinflammatory phenotype, which is embedded in the functioning of critical immune system cells in both the periphery and the brain (Hennessy et al., 2010; Nusslock and Miller, 2016). This, in turn, could underlie the increased prevalence of physical and mental health problems observed in individuals exposed to early-life adversity (Danese and McEwen, 2012; Hennessy et al., 2010; Miller et al., 2011; Raison et al., 2006). We have demonstrated that PAE alters both central and peripheral cytokine levels during early development (Bodnar et al., 2016). Specifically, at postnatal day (PN) 8, PAE animals show increased basal levels of cytokines in serum and in the hippocampus and prefrontal cortex and decreased levels in the hypothalamus and spleen. This PAE-induced alteration in cytokine balance early in life is hypothesized to underlie some of the pervasive alterations in neurobehavioral and immune function associated with PAE (for review see Bodnar and Weinberg, 2013; Drew and Kane, 2014). However, it remains to be determined if exposure to early-life adversity has a greater impact on the immune function of animals that have an altered cytokine balance early in life, such as those exposed to alcohol during gestation. Additionally, clinical research has consistently shown increased levels of plasma C-reactive protein (CRP), an acute-phase protein, in children and adults with a history of abuse and/or maltreatment (Coelho et al., 2014; Danese et al., 2008, 2011; Slopen et al., 2013). Interestingly, the CRP increase in response to early-life adversity is more prominent in individuals with current depression (Danese et al., 2008, 2011), suggesting that increased levels of CRP may be indicative of adversity-related mental health problems or may even mediate, at least in part, the negative outcomes.

Here, we combine a rodent model of PAE, which results in an early-life altered cytokine balance and increased predisposition to later life adverse health problems (Bodnar et al., 2016; Hellemans et al., 2010a; Weinberg et al., 2008; Zhang et al., 2012), with a naturalistic model of early-life adversity that replicates several aspects of the psychopathologies related to early life abuse and/or adversity in humans (Raineki et al., 2010, 2012, 2015) to examine the immune response (cytokines and CRP) of PAE animals to early-life adversity. To model early-life adversity, we provided rat mothers with insufficient bedding from PN8-12. This limited bedding environment decreases the mother's ability to construct a nest, leading to increased negative maternal behaviors (Raineki et al., 2010, 2012, 2015). Exposure to this type of early-life adversity has been shown to induce dysfunctional social behavior with the mother and peers, as well as depressive-like behavior later in life (Raineki et al., 2010, 2012, 2015; Rincón-Cortés and Sullivan, 2016; Yan et al., 2017). These behavioral deficits are, at least in part, supported by amygdala dysregulation (Raineki et al., 2010, 2012; Rincón-Cortés and Sullivan, 2016; Yan et al., 2017), data that corroborate the clinical literature demonstrating that adversity during infancy is associated with greater amygdala reactivity when exposed to emotional stimuli later in life (McCrory et al., 2013; Tottenham et al., 2011).

In rats, development of the amygdala begins prenatally, with a peak in development during the first two postnatal weeks, when there is a marked increase in neurogenesis, nuclei subdivision and neuron maturation (Bayer, 1980; Berdel et al., 1997; Berdel and Morys, 2000; Bouwmeester et al., 2002; Cunningham et al., 2002; Ryan et al., 2016; Thompson et al., 2008). As the immune system plays an important role in brain development, including development of the amygdala, and in later-life physical and mental health problems, we compared immune responses of PAE and control offspring to early-life adversity. Specifically, we evaluated the peripheral (serum) and central (amygdala) immune system responses (cytokines and CRP) of PAE animals in response to early-life adversity at PN12. Moreover, the insufficient bedding environment significantly alters how the mothers behave (Raineki et al., 2010, 2012, 2015). Because the quality of care received from the caregiver has long-lasting consequences for both physical and mental health (Champagne et al., 2003; Hofer, 1994; Rincón-Cortés and Sullivan, 2014; Weaver et al., 2004), in the present study, we evaluated the impact of the insufficient bedding environment on maternal behavior. We hypothesized that early-life adversity will differentially impact the behavior of PAE and control dams as well as the immune function of PAE and control offspring.

2. Methods

2.1. Animals and breeding

Male and female Sprague-Dawley rats were obtained from Charles River Laboratories (St. Constant, Quebec, Canada). Rats were housed with a same-sex cage mate and maintained at a constant temperature (21 ± 1 °C) and on a 12:12 light-dark cycle (lights on at 0700 h) with *ad libitum* access to water and standard laboratory chow (Harlan, Canada). After a 10-day acclimation period, males and females were paired for breeding. Vaginal smears were taken each morning, and the presence of sperm indicated gestation day 1 (G1). All experiments were performed in accordance with the National Institutes of Health (NIH) Guidelines For The Care And Use Of Laboratory Animals and the Canadian Council on Animal Care guidelines and were approved by the University of British Columbia Animal Care Committee.

2.2. Prenatal alcohol exposure

On G1, females were single-housed and randomly assigned to one of three treatment groups: alcohol, pair-fed or *ad libitum*-fed control. Dams in the alcohol group were offered *ad libitum* liquid ethanol diet with 36% ethanol-derived calories (Dyets Inc; Bethlehem, PA). The liquid ethanol diet was introduced gradually over the first 3 days with bottles containing: G1 – 66% control diet, 34% ethanol diet; G2 – 34% control diet, 66% ethanol diet; G3-21-100% ethanol diet. This diet is formulated to provide adequate nutrition to pregnant rats regardless of ethanol intake (Lan et al., 2006). Pair-fed dams were offered a liquid control diet with maltose-dextrin isocalorically substituted for ethanol, in an amount matched to the consumption of an alcohol-fed partner (g/Kg body weight/day of gestation). The control dams were offered *ad libitum* access to a pelleted form of the liquid control diet. All animals had *ad libitum* access to water, and were provided with fresh diet daily within 1 h of lights off to prevent a shift in corticosterone circadian rhythms, which occurs in animals that are on a restricted feeding schedule, such as the pair-fed dams (Gallo and Weinberg, 1981; Krieger, 1974). Experimental diets were continued through G21. Beginning on G22, all animals were offered *ad libitum* access to standard laboratory chow and water, which they received throughout lactation. Pregnant dams were left undisturbed except for cage changing on G1, G7, and G14. On the day of birth (postnatal day 1 – PN1), litters were culled to 12 pups with an attempt to preserve an equal number of males and females per litter, and transferred to a clean cage.

While a pair-fed group was included in the initial study design, specific pair-feeding effects are analyzed and presented separately in the Supplementary section. Historically, the pair-fed group was introduced to account for the decreased food intake associated with chronic alcohol consumption, with the goal of separating alcohol effects from those of undernutrition. However, adverse

effects of alcohol per se on a vast number of outcomes are now well established. Moreover, pair-feeding is a confounded "control" group: 1) It can never account for the nutritional effects associated with alcohol consumption, including alterations in absorption and utilization of nutrients (Weinberg, 1984) and increases in satiety (Lin et al., 1998); 2) Because pair-fed animals receive a reduced food ration, less than they would consume if allowed to eat *ad libitum*, they generally consume their entire day's ration within a few hours, and are thus essentially food deprived until next feeding (Gallo and Weinberg, 1981; Weinberg, 1984). Not only is this an abnormal feeding pattern, but pair-feeding also introduces a mild prenatal stress component, which in itself may have a long-term impact on offspring developing neurobiological systems, including the neuroendocrine axis (Vieau et al., 2007).

2.3. Early-life adversity

Within 2 h of lights off on PN7, half the dams/litters from each group (PAE, n = 16; pair-fed, n = 16; or *ad libitum*-fed control, n = 15) were transferred to clean cages with limited nesting/bedding material that consisted of 300 mL of Beta Chip® bedding (Northeastern Products Corp, Warrensburg, NY). The animals remained in this limited bedding environment until the afternoon of PN12 (within 2 h of lights off). The remaining dams/litters from each group (PAE, n = 15; pair-fed, n = 15; or *ad libitum*-fed control, n = 17) were transferred to clean cages with abundant nesting/bedding material (3000 mL) within 2 h of lights off on PN7 and remained in this environment until the afternoon of PN12.

2.4. Maternal behavior observations

Observations of maternal behaviors occurred 3 times per day from postpartum day 8 through 12. Each observation consisted of a 75-min period during which each dam was observed once every 3 min for the following behaviors: licking and grooming (anogenital licking and body grooming were not distinguished), nursing [arched-back, blanket, passive (nursing while supine or on side)], and self-directed behaviors (eating, drinking, self-grooming, sleeping, and exploring cage). Negative maternal behaviors were scored if dams were stepping on pups, dragging pups (i.e., moving around the cage if pups remained attached to the nipples), and handling pups roughly, as previously described (Workman et al., 2015; Raineki et al., 2012, 2015). Additionally, the frequency of pups' vocalizations was also recorded.

2.5. Lag sequential analysis of maternal behavior

We used Noldus Observer 5.0 to calculate the frequency of transitions between pairs of behaviors in order to determine how often a behavior is immediately followed by the same behavior (consistency). Analysis was performed on transitions between the five behavior categories: nursing (arched-back, blanket, and passive), self-directed (eating, drinking, self-grooming, sleeping, and exploring cage), negative (stepping on pups, dragging pups, and handling pups roughly), other (nest building and retrieving pups), and licking and grooming, creating a possibility of 25 combined transitions.

2.6. Blood and brain collection

On PN12, at least one male and/or female pup per litter was decapitated and trunk blood and brains were collected. Blood was centrifuged and serum was collected and stored at −80 °C. Brains were removed and placed in a rodent brain matrix (ASI Instruments, Warren, MI) and coronal sections were cut every 1000 μm from the rostral to caudal end. The sections were placed on glass slides on dry ice, allowing sections to freeze mount to the slides. The sections starting at approximately bregma −2.04 mm (Paxinos and Watson, 2005) were used to dissect the amygdala using Harris Uni-Core disposable micro-punches (2 mm, Sigma-Aldrich, ON, Canada). Amygdala micro-punches from both sides were deposited into 2 mL tubes and stored at −80 °C until homogenization.

2.7. Amygdala homogenization

The amygdala from each pup was homogenized in tubes containing 8 zirconium oxide beads and 200 μL of cold lysis buffer using an Omni Bead Ruptor 24 (Omni International, Kennesaw, GA) in 4 cycles (speed: 3.10, time: 6 sec), with 1 min on ice between cycles. Homogenates were centrifuged and separate aliquots of supernatant removed for protein and cytokine analysis, and stored at −20 °C until assayed.

2.8. Multiplex cytokine and CRP immunoassays

Cytokine assays were performed using the Meso Scale Discovery (MSD) proinflammatory panel 1 rat V-PLEX kit, which allows for the simultaneous measurement of IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-13, IFN-γ, KC/GRO (CXCL1), and TNF-α (MSD, Rockville, MD – Note: IL-2 was only measured in serum samples as it was recently removed from the V-PLEX panel). Samples were diluted (1:4 for serum, 1:2 for amygdala) in diluent 42. Serum assays were performed using the standard MSD protocol. The following adjustments to the protocol were made for the amygdala samples to increase assay sensitivity: The sample/standard volume was doubled (100 μL added to each well) and after sample addition, plates were incubated overnight (4 °C, on plate shaker) and washed following 18 h incubation. CRP levels were detected using antibody pairs (catalog #: DY1744, R&D Systems, Minneapolis, MN), with capture antibodies printed in the wells of standard-bind multiplex assay plates (MSD proprietary printing service). Serum samples were diluted in PBS + 1% BSA (1:50,000 dilution), followed by a 1:2 dilution in diluent 42. Detection antibodies were derivatized with electrochemiluminescent SULFO-Tag NHS-ester by MSD and 2.88 μl SULFO-TAG labeled Streptavidin was added, with the detection antibody, according to standard MSD protocol. Cytokine and CRP plates were read using a Sector Imager 2400 (MSD) and data analyzed using the MSD Discovery Workbench software v. 4.0 (MSD). The following lower limit of detection (LLOD) ranges were observed (pg/mL) – IL-1β: 12.2–30.7; IL-2: 71.3; IL-4: 0.06–0.31; IL-5: 8.93–24.1; IL-6: 15.2–29.3; IL-10: 1.83–3.38; IL-13: 0.68–1.81; IFN-γ: 0.924–1.22; TNF-α: 0.291–1.27; KC/GRO: 0.06–4.20; CRP: 4.89.

Total protein levels were quantified in the amygdala homogenates using the Pierce Microplate BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Tissue homogenate samples were run in quadruplicate to determine the average protein concentration. Amygdala cytokine levels were adjusted and reported as pg cytokine/mg protein and serum cytokine and CRP levels were reported as pg/mL and μg/mL, respectively.

2.9. Statistical analyses

All data are expressed as mean ± SEM. All data were analyzed by two-way ANOVA (for maternal behavior: alcohol consumption and bedding as factors; for remaining analyses: prenatal treatment and rearing condition as factors). When significant, ANOVAs were followed by Newman–Keuls *post hoc* tests. Outliers were identified and removed using the Robust regression and Outlier removal (ROUT) method with Q = 0.05. Cytokine and CRP levels were not normally distributed and were thus Blom transformed for statisti-

cal analysis. Untransformed data are presented, for clarity. Further analyses utilized planned pairwise comparisons to test the *a priori* hypotheses that: 1) PAE will alter immune function compared to that in controls; and 2) early-life adversity will differentially alter PAE and control immune function. In all cases, differences were considered significant when $p \leq 0.05$.

3. Results

3.1. Maternal behavior

Neither alcohol consumption during pregnancy nor exposure to insufficient bedding from PN8-12 significantly altered the frequency of total nursing (Fig. 1A). However, analysis of the different nursing postures indicated that all mothers exposed to the insufficient bedding environment decreased their frequency of arched-back nursing and increased their frequency of blanket nursing [Fig. 1B and C; significant main effects of bedding were detected for arched-back nursing ($F_{(1,59)} = 19.82$, $p < 0.0001$) and blanket nursing ($F_{(1,59)} = 6.89$, $p < 0.01$)] with no change in frequency of passive nursing (data not shown). Moreover, the frequencies of licking and grooming and self-directed behaviors were not affected by either alcohol consumption or exposure to insufficient bedding (Fig. 1D and E). Exposure to insufficient bedding significantly increased the frequency of negative maternal behaviors in all mothers, independent of alcohol consumption during pregnancy [Fig. 1F; significant main effect of bedding was detected for negative maternal behaviors ($F_{(1,59)} = 109.76$, $p < 0.0001$)]. Finally, analysis of the maternal behavior sequence indicated that all mothers exposed to the insufficient bedding environment were significantly less consistent in their behavioral patterns (Fig. 1G); that is, they were more likely to change behaviors between consecutive observations [significant main effect of bedding was detected for consistency ($F_{(1,59)} = 10.68$, $p < 0.002$)].

3.2. Pup vocalization

PAE pups in the normal rearing (abundant bedding) environment vocalized significantly less than normally reared control pups (Fig. 2). Exposure to adverse rearing increased vocalization in both groups, but PAE pups showed a significantly smaller increase compared to control pups [significant main effects of prenatal treatment ($F_{(1,59)} = 52.55$, $p < 0.0001$) and rearing condition ($F_{(1,59)} = 221.32$, $p < 0.0001$), and significant interaction between prenatal treatment and rearing condition ($F_{(1,59)} = 12.91$, $p < 0.0007$) were detected for vocalization].

3.3. Serum CRP

Serum CRP levels were increased at PN12 in PAE compared to control pups, independent of rearing condition (Fig. 3). Moreover, exposure to adverse rearing resulted in an increase in serum CRP levels in both groups [significant main effects of prenatal treatment ($F_{(1,48)} = 6.98$, $p < 0.01$) and rearing condition ($F_{(1,59)} = 6.08$, $p < 0.02$) were detected for CRP].

3.4. Serum cytokines

Exposure to adverse rearing from PN8-12 significantly reduced serum levels of KC/GRO, IL-10 and TNF-α in control but not PAE

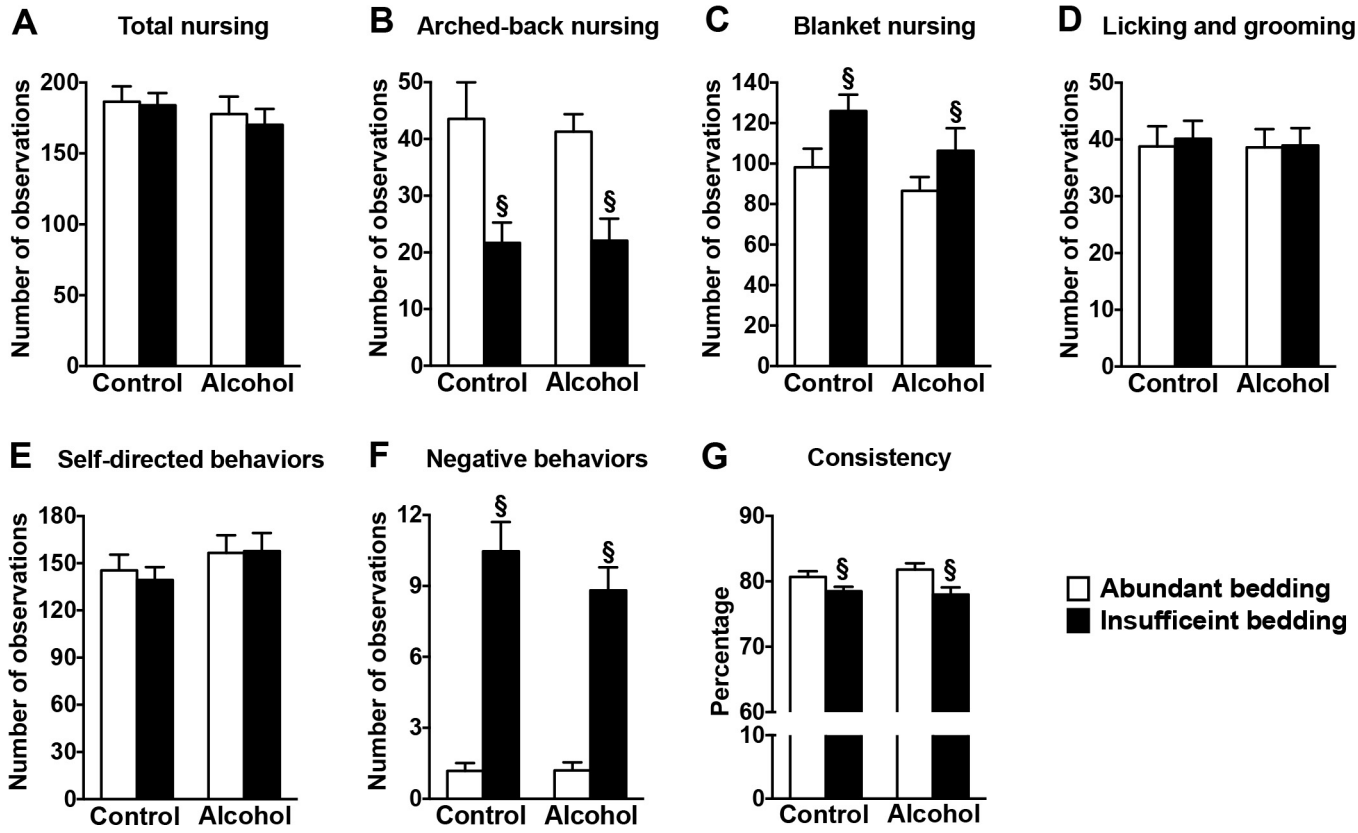

Fig. 1. Maternal behavior of mothers that consumed alcohol during pregnancy and were exposed to insufficient bedding for nest building. Bars represent mean ± SEM of total nursing (A), arched-back nursing (B), blanket nursing (C), passive nursing (D), licking and grooming (E), self-directed behaviors (F), negative maternal behaviors (G), and behavioral consistency (H) from PN8-12. § indicates significant main effect of bedding, where all mothers exposed to insufficient bedding were different from mothers exposed to abundant bedding, independent of gestational group (n = 15–17 for all groups).

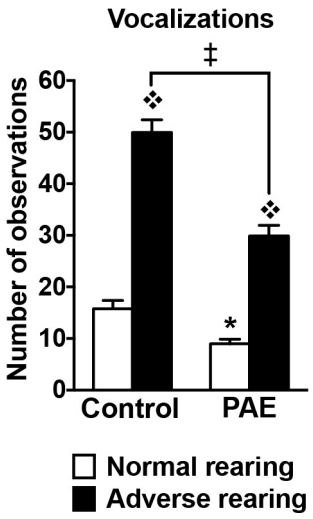

Fig. 2. Vocalization of pups that were exposed to alcohol during gestation and/or were exposed to early-life adversity. Bars represent mean ± SEM of total number of vocalizations from PN8-12. * indicates that normally reared PAE pups vocalized less than normally reared control pups; ❖ indicates that PAE and control pups normally reared vocalized less than PAE and control pups exposed to adverse rearing; ‡ indicates that PAE pups exposed to adverse rearing vocalized less than control pups exposed to adverse rearing (n = 15–17 for all groups).

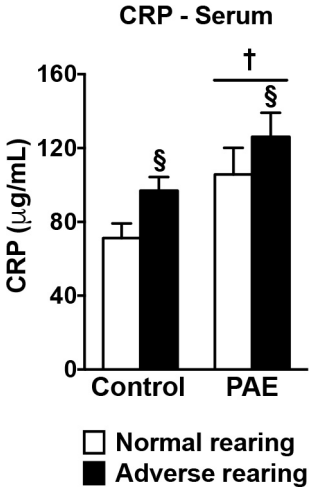

Fig. 3. Serum CRP levels in pups that were exposed to alcohol during gestation and/or were exposed to early-life adversity. Bars represent mean ± SEM of serum CRP levels at PN12. § indicates significant main effect of rearing condition, where all pups exposed to adverse rearing showed higher CRP levels than pups exposed to normal rearing, independent of prenatal treatment; † indicates significant main effect of prenatal treatment, where all PAE pups showed higher CRP levels than control pups, independent of rearing condition (n = 12–14 for all groups).

pups [Fig. 4A–C; no significant interaction between prenatal treatment and rearing condition was detected for KC/GRO, *a priori* analysis for KC/GRO comparing control normal rearing to control adverse rearing ($p = 0.05$); significant interaction between prenatal treatment and rearing condition was detected for IL-10 ($F_{(1,46)} = 3.86$, $p = 0.05$), and TNF-α ($F_{(1,44)} = 4.08$, $p < 0.05$); *a priori* analysis for IL-10 and TNF-α comparing control normal rearing to control adverse rearing ($p = 0.05$ and $p < 0.05$, respectively)]. However, serum levels of IFN-ɣ were reduced in all pups' exposed to adverse rearing [Fig. 4D; significant main effect of rearing condition was detected for IFN-ɣ ($F_{(1,48)} = 6.68$, $p < 0.01$)]. There were no effects of PAE or adverse rearing on serum levels of IL-13 (data not shown).

Serum levels of IL-4, IL-5 and IL-2 were low or below the limit of detection for one or more groups and therefore not analyzed statistically (Fig. 4E–G). However, visual inspection of the graphs indicates that the pattern of IL-4 levels was similar to the pattern of KC/GRO, IL-10 and TNF-α. Indeed, while levels of IL-4 in control pups exposed to adverse rearing were undetectable, 42% of normally reared control pups, 46% of normally reared PAE pups, and 67% of PAE pups exposed to early-life adversity showed detectable levels of IL-4 in serum. Moreover, serum levels of IL-5 were undetectable in both control and PAE pups exposed to adverse rearing, while 50% of control and 46% of PAE pups normally reared showing detectable levels of IL-5 in serum. Finally, levels of IL-2 were undetectable in control pups exposed to early-life adversity, but 61% of control pups normally reared showed detectable levels of IL-2. By contrast, IL-2 levels were undetectable in normally reared PAE pups and detectable in only 10% of pups exposed to early-life adversity. Serum levels of IL-6 and IL-1β were below the limit of detection for most of the animals and were not analyzed or shown.

3.5. Amygdala cytokines

PAE pups showed a significant reduction in amygdala levels of KC/GRO compared to control pups, independent of rearing condition (Fig. 5A). However, exposure to adverse rearing from PN8-12 significantly increased amygdala levels of KC/GRO in both groups [significant main effects of prenatal treatment ($F_{(1,48)} = 18.38$, $p < 0.0001$) and rearing condition ($F_{(1,48)} = 7.48$, $p < 0.009$) were detected for KC/GRO]. Moreover, normally reared PAE pups showed a significant increase in amygdala levels of IL-10 compared to their control counterparts [Fig. 5B; no significant interaction between prenatal treatment and rearing condition was detected for IL-10, *a priori* analysis for IL-10 comparing control normal rearing to PAE normal rearing ($p = 0.05$)]. There were no effects of PAE or adverse rearing on amygdala levels of TNF-α, IFN-ɣ, IL-4, IL-5, IL-1β (Fig. 5C–G), nor IL-6 (data not shown). Amygdala levels of IL-13 were below the limit of detection for most of the animals and were not analyzed or shown.

4. Discussion

The present results indicate that PAE alters immune system development and alters both behavioral and immune responses to early-life adversity. Altered immune system development following PAE was reflected by increased CRP levels in serum, as well as by reduced KC/GRO and increased IL-10 levels in the amygdala at PN12. Moreover, exposure to early-life adversity differentially affected the immune system of PAE and control animals. While PAE and control pups showed similar reductions in serum levels of IFN-ɣ and IL-5 in response to early-life adversity, PAE animals failed to show the adversity-induced reductions in serum KC/GRO, TNF-α, IL-10, and IL-4 observed in control animals. The lack of response to early-life adversity in PAE animals was also observed behaviorally, as PAE pups vocalized less than controls when exposed to early-life adversity. These differential behavioral and immune responses occurred despite exposure of PAE and control pups to the same level of negative maternal behavior in the insufficient bedding environment. A possible limitation of the current study is the correlational nature of the experimental design. However, despite being correlational, here we present compelling evidence that PAE animals do not respond in the same way as controls to early-life adversity. Moreover, there are very few clinical and preclinical studies that investigate the dynamic and overlapping effects of PAE and early-life adversity (Alberry and Singh, 2016; Price et al., 2017) and the current study starts to fill this gap in the literature by laying the groundwork for future studies.

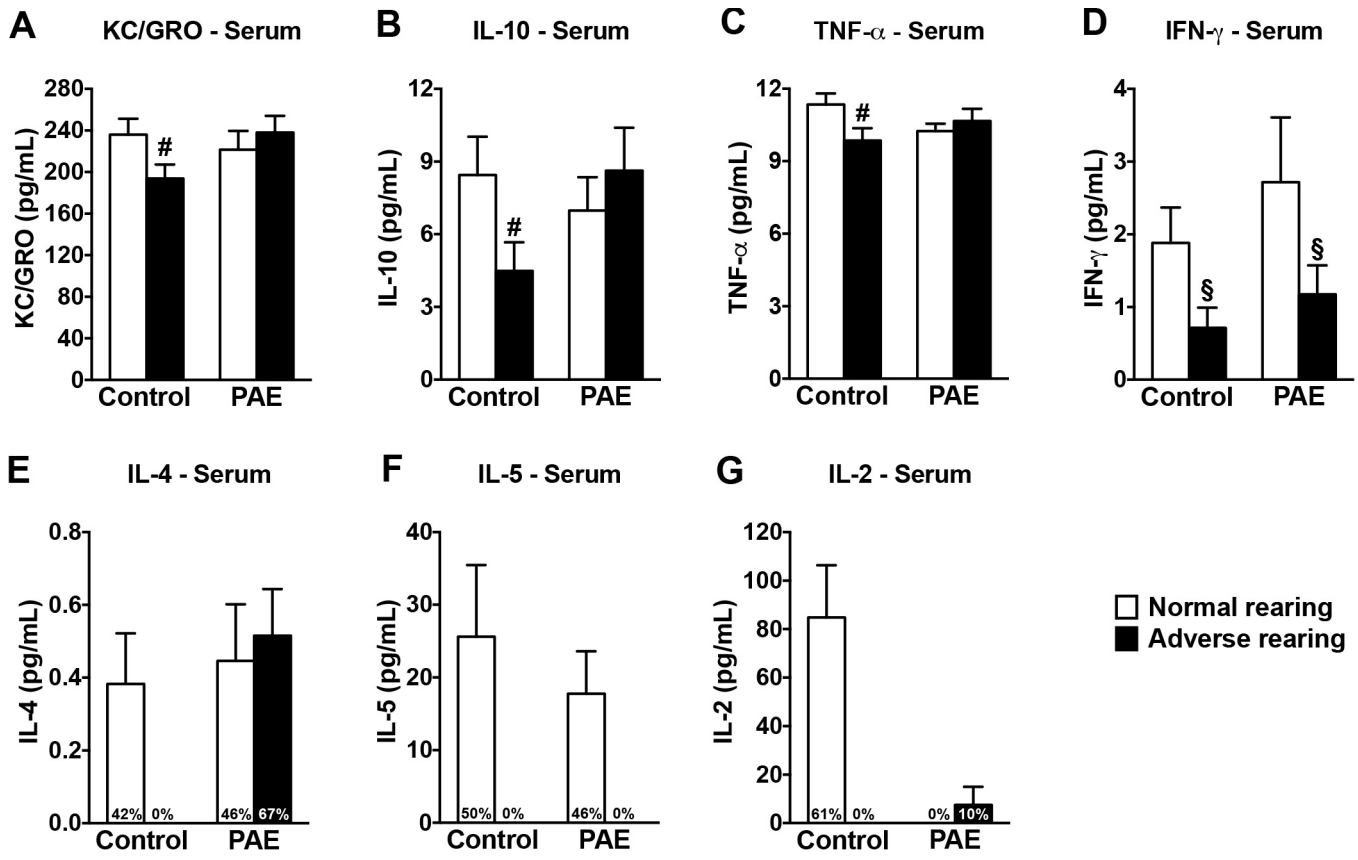

Fig. 4. Serum cytokine levels in pups that were exposed to alcohol during gestation and/or were exposed to early-life adversity. Bars represent mean ± SEM of serum KC/GRO (A), IL-10 (B), TNF-α (C), IFN-γ (D), IL-4 (E), IL-5 (F), and IL-2 (G) levels at PN12. § indicates significant main effect of rearing condition, where all pups exposed to adverse rearing showed lower IFN-γ levels than pups exposed to normal rearing, independent of prenatal treatment; # indicates that control pups exposed to adverse rearing showed lower KC/GRO, IL-10, and TNF-α levels than control pups exposed to normal rearing based on *a priori* comparison (n = 11–13 for all groups, excluding IL-4, IL-5 and IL-2 that were not analyzed statistically).

4.1. Similar maternal behavior response to insufficient bedding in alcohol consuming and control mothers, but differential effects in the offspring

Our assessment of maternal behavior indicates that mothers consuming alcohol during gestation did not behave differently from control mothers when exposed to an environment with insufficient nest bedding material. Indeed, replicating previous findings (Raineki et al., 2010, 2012, 2015), all mothers that were exposed to insufficient bedding increased their expression of negative maternal behaviors. However, exposure to insufficient bedding did not alter the frequency of licking and grooming or self-directed behaviors in PAE and control dams. In the rat, variations in maternal behavior, particularly licking and grooming, have been shown to modulate the development of offspring endocrine, emotional and cognitive responses to stress (Champagne et al., 2003; Liu et al., 1997) Thus our findings strongly suggest that the altered endocrine and behavioral responses to stressors that are consistently observed in PAE offspring (Hellemans et al., 2010a; Schneider et al., 2011; Valenzuela et al., 2012; Weinberg et al., 2008) are primarily due to the effects of alcohol exposure and not to altered maternal behavior. Additionally, exposure to insufficient bedding did not alter the frequency of total nursing in PAE and control dams. Nevertheless, we observed changes in the type of nursing behavior as mothers exposed to insufficient bedding reduced arched-back and increased blanket nursing. The increased negative maternal behavior and altered nursing behavior style observed in mothers exposed to insufficient bedding suggests a reduction in the quality of maternal care. Preclinical and clinical research has consistently shown that the quality of care received from the caregiver has long-lasting consequences for neurobehavioral development of the offspring (Bowlby, 1969; Champagne et al., 2003; Heim et al., 2010; Hofer, 1994; Rincón-Cortés and Sullivan, 2014; Weaver et al., 2004). Accordingly, the changes in maternal behavior observed in mothers exposed to insufficient bedding might play an important role in the altered behavior and immune function of their offspring.

Exposure to insufficient bedding also resulted in more inconsistent maternal behavior. Specifically, each type of maternal behavior was more frequently followed by a different type rather than the same type of behavior, suggesting that dams in the insufficient bedding environment showed less consistent maternal care than those with abundant bedding. This inconsistency in maternal behavior introduces instability to an otherwise extremely stereotyped behavioral repertoire, altering the expected and required pattern of sensory stimulation received by the infant during a critical period of development. Inconsistency in maternal behavior has been previously observed using a more extreme early-life adversity model where the mother and litter are housed in a cage with a mesh bottom and provided with a single paper towel for nest building (Ivy et al., 2008; Molet et al., 2016). Importantly, the combination of increased negative maternal behavior, altered nursing behaviors, and inconsistent maternal behavior indicates that changes in maternal care were not limited to reductions in quantity and quality, but also to increased inconsistency in the pattern of care received from the caregiver in response to the insufficient

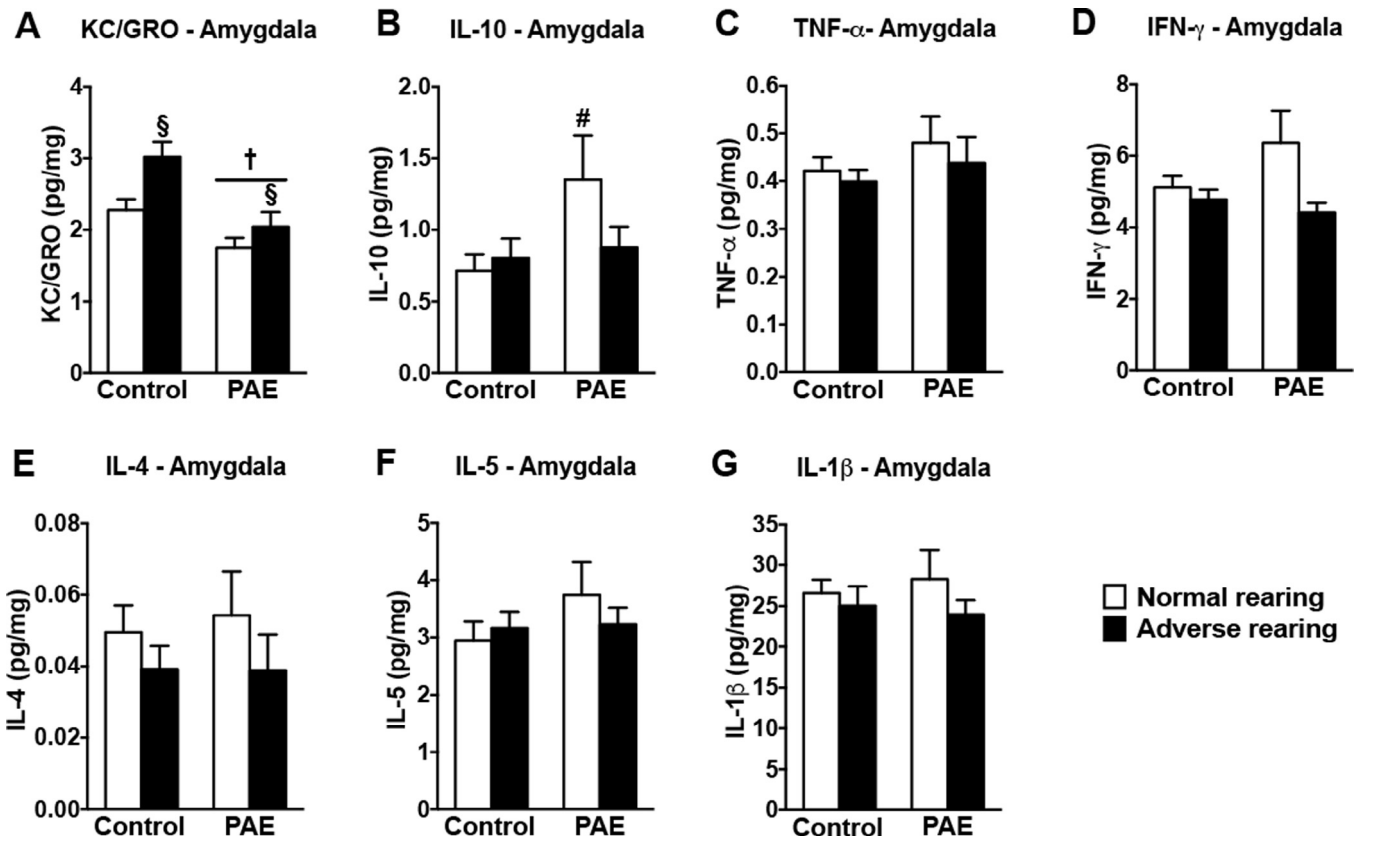

Fig. 5. Amygdala cytokine levels in pups that were exposed to alcohol during gestation and/or were exposed to early-life adversity. Bars represent mean ± SEM of amygdala KC/GRO (A), IL-10 (B), TNF-α (C), IFN-γ (D), IL-4 (E), IL-5 (F), and IL-1β (G) levels at PN12. § indicates significant main effect of rearing condition, where all pups exposed to adverse rearing showed higher KC/GRO levels than pups exposed to normal rearing, independent of prenatal treatment; † indicates significant main effect of prenatal treatment, where all PAE pups showed higher KC/GRO levels than control pups, independent of rearing condition; # indicates that PAE pups exposed to normal rearing showed higher IL-10 levels than control pups exposed to normal rearing based on *a priori* comparisons (n = 11–15 for all groups).

bedding environment. These changes in the quantity, quality and pattern of maternal behavior observed in response to the insufficient bedding environment more closely replicate human findings where, in general, the abusive caregiver not only displays increased levels of negative/abusive behaviors but does so in an unpredictable manner (Gaudin et al., 1996).

Findings in the present study are consistent with but do not fully replicate our previous data, which showed that alcohol consumption during pregnancy resulted in small but significant reductions in total nursing and increases in self-directed behaviors (Workman et al., 2015). It is likely that differences in the observation periods between the two studies could account for the difference in results. In the previous experiment, maternal behavior observations were spread across the first 3 weeks postpartum, whereas maternal observations in the current experiment were concentrated between PN8-12. Importantly, both studies found that alcohol consumption during gestation did not alter the frequency of licking and grooming.

Corroborating previous findings indicating that pups prenatally exposed to alcohol show reduced ultrasonic vocalizations (Barron et al., 2000; Kehoe and Shoemaker, 1991), our results indicate that PAE pups vocalized less than control pups. Mother-infant interactions are dyadic, requiring a fine coordination of behaviors between the mother and infant (Bowlby, 1969). Failure to display an expected behavior or to respond to a behavior by the mother and/or pup can negatively impact mother-infant interactions and attachment. Vocalization, especially ultrasonic vocalization in rats, is used by pups to elicit maternal care or to indicate physical distress or separation from the mother (Hofer, 1996). The reduction in vocalization in PAE pups suggests that PAE pups are not able to perceive and/or respond appropriately to environmental stimuli or cues, which may impact mother-infant attachment. Importantly, this reduction in vocalization in PAE pups occurred despite PAE and control pups receiving similar maternal care. Moreover, consistent with previous findings (Blaze et al., 2013; Raineki et al., 2010, 2012, 2015; Yan et al., 2017), exposure to early-life adversity increased pup vocalization across prenatal groups. However, this response to early-life adversity was significantly reduced in PAE compared to control pups, suggesting that even when exposed to an adverse environment, PAE pups show a deficit in perceiving and/or responding to environmental stimuli.

4.2. The immune function of PAE and control animals are differentially affected by early-life adversity

Adverse rearing from PN8-12 resulted in an overall suppression of immune function in control animals, as they showed reductions in serum levels of both pro- and anti-inflammatory cytokines. Specifically, control animals exposed to early-life adversity showed reduction in serum levels of KC/GRO, TNF-α, IFN-γ, IL-2, IL-4, IL-5, and IL-10 at PN12. Our results are consistent with previous studies in non-human primates and rodents demonstrating that, in the short-term, exposure to chronic early-life adversity – such as maternal separation – results in suppression of immune function (Coe et al., 1987; Dimatelis et al., 2012; Ganguly and Brenhouse, 2015; Laudenslager et al., 1982; Roque et al., 2016). Indeed, in rodents, maternal separation has been shown to decrease the number of microglial cells in the ventral tegmental area and substantia

nigra (Chocyk et al., 2011) and, similar to our peripheral results, resulted in decreased central (IL-1β and IL-10) and peripheral (IL-1β and IL-6) cytokine levels (Dimatelis et al., 2012; Roque et al., 2016). This adversity-related suppression of immune function in infancy appears to sensitize the immune system in the long-term, resulting in an enhanced proinflammatory phenotype in later life, both in the periphery and the brain (Ganguly and Brenhouse, 2015; Hennessy et al., 2010; Nusslock and Miller, 2016; Pinheiro et al., 2015; Réus et al., 2013). Importantly, the preclinical and clinical literature has consistently shown that a chronic proinflammatory bias is associated with increased vulnerability to diseases/disorders later in life, including infections, cardiovascular disease, diabetes, cognitive and behavioral problems and mental health disorders (Bauman et al., 1997; Bilbo and Schwarz, 2009; Danese and McEwen, 2012; Meyer et al., 2009; Miller et al., 2011; Raison et al., 2006). Not surprisingly, these same diseases/disorders are highly prevalent in individuals that encounter adversity during infancy, and have been replicated using animal models (Chen and Baram, 2016; Cirulli et al., 2009; Danese and McEwen, 2012; Drury et al., 2016; Heim et al., 2010; McEwen, 2008; Raineki et al., 2012; Teicher et al., 2003). We have shown previously that pups exposed to the naturalistic early-life adversity model used here exhibit dysfunctional social attachment behaviors with the mother in infancy (Raineki et al., 2010). This dysfunctional social behavior with the mother is followed by deficits in social behavior with peers, as well as depressive-like behavior later in life (Raineki et al., 2012, 2015; Yan et al., 2017). As cytokines are potent neuromodulators during development (Bajetto et al., 2001; Bessis et al., 2005; Deverman and Patterson, 2009; Smith et al., 2007; Stephan et al., 2012), the short- and long-term effects of early-life adversity may have their origins in the altered serum cytokine levels during infancy.

Animals exposed to alcohol during gestation did not show the same suppression of immune function in response to early-life adversity observed in controls. Indeed, PAE pups exposed to early-life adversity failed to show the reduction in serum levels of KC/GRO, TNF-α, IL-4, and IL-10 observed in controls, suggesting differential peripheral immune responses of PAE and controls to early-life adversity. This inappropriate lack of responsivity to early-life adversity may lead to differential neurodevelopment, and subsequent alterations in behavioral and immune/physiological responses. In support of this, we observed reduced levels of vocalization in PAE pups exposed to early-life adversity compared to that in their control counterparts. Moreover, abnormal immune responses in PAE animals – either blunted or potentiated – are commonly observed following several different challenges in adulthood. Specifically, the lipopolysaccharide (LPS)-induced febrile response is blunted in PAE animals (Taylor et al., 1999; Yirmiya et al., 1993), possibly due to a decreased hypothalamic IL-1β response to LPS (Taylor et al., 1999). Additionally, PAE animals show reduced TNF-α responses to an immune (LPS) challenge (Chiappelli et al., 1997). By contrast, exposure to repeated restraint stress induced an exacerbated plasma proinflammatory cytokine (IL-1β, TNF-α, IL-6) response to LPS challenge (Zhang et al., 2005). Finally, PAE animals show a more severe and prolonged course of inflammation in an adjuvant-induced arthritis model (Zhang et al., 2012). Together, these data suggest that exposure to alcohol during gestation alters development of the immune system, resulting in altered/inappropriate immune responses that may be unmasked particularly when the system is challenged later in life.

Serum CRP levels were increased in control animals exposed to early-life adversity. These results are in line with clinical findings showing that individuals with a history of abuse and/or maltreatment have increased levels of plasma CRP (Coelho et al., 2014; Danese et al., 2008, 2011; Slopen et al., 2013). Interestingly, increased CRP levels following early-life adversity are more pronounced in individuals with current depression (Danese et al., 2008, 2011), suggesting that increased levels of CRP may be indicative of adversity-related mental health problems or may even mediate, at least in part, the negative outcomes. We suggest that, similar to the clinical findings, increased CRP levels may, at least in part, underlie the increased levels of offspring depressive-like behavior observed in our previous studies using this model of early-life adversity (Raineki et al., 2012, 2015; Yan et al., 2017). Additionally, in the present study, PAE animals showed increased CRP levels compared to control animals, and PAE pups exposed to early-life adversity showed even higher levels of serum CRP. This CRP profile may underlie the increased vulnerability to anxiety- and depressive-like behaviors observed in these animals (Brocardo et al., 2012; Hellemans et al., 2008, 2010a,b; Raineki et al., 2016), consistent with the clinical literature indicating that individuals exposed to alcohol during gestation show increased vulnerability to mental health problems when compared to controls (Famy et al., 1998; O'Connor and Kasari, 2000; Pei et al., 2011). Moreover, it has been proposed that exposure to adversity and/or stress has a greater impact on individuals with PAE than on unexposed individuals, resulting in an even higher rate of mental health problems in that population (Hellemans et al., 2010a; McLachlan et al., 2016; Raineki et al., 2014). Indeed, we have previously demonstrated that PAE rats exposed to chronic, unpredictable but mild stressful experiences in adulthood show increased anxiety- and depressive-like behaviors (Hellemans et al., 2008, 2010a,b).

4.3. Amygdala immune function in PAE and control animals in response to early-life adversity

The amygdala is one of the most important neural substrates regulating stress, fear, and emotional responses; indeed, dysregulation of amygdala structure and function is implicated in the pathophysiology of many mental health problems (Drevets et al., 2008; Krishnan and Nestler, 2010; Nestler et al., 2002; Ressler and Mayberg, 2007; Tottenham, 2012). The amygdala starts developing prenatally, but its development is considered protracted and extends into adolescence. The peak of amygdala neurogenesis, nuclei subdivision and neuron maturation occurs in the first two postnatal weeks (Bayer, 1980; Berdel et al., 1997; Berdel and Morys, 2000; Bouwmeester et al., 2002; Cunningham et al., 2002; Ryan et al., 2016; Thompson et al., 2008), when amygdala-dependent behaviors undergo marked development (Raineki et al., 2009; Sullivan et al., 2000; Thompson et al., 2008). Importantly, exposure to early-life adversity during this critical period negatively alters the amygdala's neurodevelopmental trajectory, resulting in increased amygdala reactivity later in life (McCrory et al., 2013; Raineki et al., 2012, 2015; Tottenham, 2012; Tottenham et al., 2011), which may underlie many of the mental health problems observed in those individuals (Chen and Baram, 2016; Cirulli et al., 2009; Danese and McEwen, 2012; Drury et al., 2016; Heim et al., 2010; McEwen, 2008; Raineki et al., 2012; Teicher et al., 2003). Here, we observed an increase in KC/GRO in the amygdala of control animals exposed to early-life adversity, suggesting that exposure to early-life adversity may induce neuroinflammation in the amygdala. KC/GRO is a chemokine involved in the recruitment and activation of neutrophils in the brain (Johnson et al., 2011; Shaftel et al., 2007), which is an early component of neuroinflammation. The adversity-induced neuroinflammation observed here likely mediates some of the long-lasting amygdala structural and functional deficits observed following early-life adversity, as it occurs during a critical period for amygdala maturation.

The clinical literature indicates that PAE also affects amygdala development, as children exposed to alcohol during gestation show

reduced amygdala volume in the first postnatal week (Donald et al., 2016). Furthermore, animal models of PAE have demonstrated the deleterious effects of PAE on amygdala structure and function in adulthood (Cullen et al., 2013; Raineki et al., 2014). Importantly, PAE-related alterations in the amygdala have been associated with increased depressive- and anxiety-like behaviors (Hellemans et al., 2008, 2010a,b; Cullen et al., 2013; Raineki et al., 2014). The current data indicate that PAE resulted in reduced KC/GRO and increased IL-10 in the amygdala. IL-10 is an anti-inflammatory cytokine that is believed to have neuroprotective effects (Rodts-Palenik et al., 2004; Thompson et al., 2013). The increase in amygdala IL-10 levels observed in PAE rats at PN12 may be a compensatory response to counter the deleterious effects of the altered cytokine balance observed in PAE pups (Bodnar et al., 2016). IL-10 is an important regulator of chemokine expression in response to inflammation, including KC/GRO (Kim et al., 1998; Shanley et al., 2000). To this end, the reduced levels of KC/GRO in the amygdala of PAE animals may be a response to the IL-10 increase. Of note, overexpression of IL-10 during development in the absence of inflammation can negatively alter development and result in behavioral abnormalities in adulthood (Meyer et al., 2008). Alternatively, if the increase of the anti-inflammatory cytokine IL-10 in the amygdala of PAE animals is not a response to a previous proinflammatory surge at PN8 (Bodnar et al., 2016), it may not have the expected beneficial effects, as none of the proinflammatory cytokines analyzed at PN12 were elevated in the amygdala of PAE animals.

4.4. Implications and conclusions

The power of the two animal models utilized in the present study is that they allow us to begin to identify independent and interactive effects of PAE and early-life adversity. We demonstrate altered behavioral and immune responses of PAE pups to early-life adversity, which may have profound downstream effects on ongoing neurobehavioral development and further exacerbate the negative effects of PAE. Identifying the unique ways in which adverse experiences during infancy impact individuals prenatally exposed to alcohol is a critical step towards establishing more targeted interventions and/or treatments for affected individuals. In particular, the dysregulated immune function following PAE and/or early-life adversity indicates that intervention strategies that target immune function may be a novel therapeutic approach for affected individuals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbi.2017.07.001.

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