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Sex-specific deficits in biochemical but not behavioral responses to delay fear conditioning in prenatal alcohol exposure mice

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Abstract

Background: Studies in clinical populations and preclinical models have shown that prenatal alcohol exposure (PAE) is associated with impairments in the acquisition, consolidation and recall of information, with deficits in hippocampal formation-dependent learning and memory being a common finding. The glucocorticoid receptor (GR), mineralocorticoid receptor (MR), and extracellular signal-regulated kinase 2 (ERK2) are key regulators of hippocampal formation development, structure and functioning and, thus, are potential mediators of PAE's effects on this brain region. In the present studies, we employed a well-characterized mouse model of PAE to identify biochemical mechanisms that may underlie activity-dependent learning and memory deficits associated with PAE.

Methods: Mouse dams consumed either 10% (w/v) ethanol in 0.066% (w/v) saccharin (SAC) or 0.066% (w/v) SAC alone using a limited (4-h) access, drinking-in-the-dark paradigm. Male and female offspring (~180-days of age) were trained using a delay conditioning procedure and contextual fear responses (freezing behavior) were measured 24 h later. Hippocampal formation tissue and blood were collected from three behavioral groups of animals: 20 min following conditioning (conditioning only group), 20 min following the re-exposure to the context (conditioning plus re-exposure group), and behaviorally naïve (naïve group) mice. Plasma corticosterone levels were measured by enzyme immunoassay. Immunoblotting techniques were used to measure protein levels of the GR, MR, ERK1 and ERK2 in nuclear and membrane fractions prepared from the hippocampal formation.

Results: Adult SAC control male and female mice displayed similar levels of contextual fear. However, significant sex differences were observed in freezing exhibited during the conditioning

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session. Compared to same-sex SAC controls, male and female PAE mice demonstrated context fear deficits. While plasma corticosterone concentrations were elevated in PAE males and females relative to their respective SAC naïve controls, plasma corticosterone concentrations in the conditioning only and conditioning plus re-exposure groups were similar in SAC and PAE animals. Relative to the respective naïve group, nuclear GR protein levels were increased in SAC, but not PAE, male hippocampal formation in the conditioning only group. In contrast, no difference was observed between nuclear GR levels in the naïve and conditioning plus re-exposure groups. In females, nuclear GR levels were significantly reduced by PAE but there was no effect of behavioral group or interaction between prenatal treatment and behavioral group. In males, nuclear MR levels were significantly elevated in the SAC conditioning plus re-exposure group compared to SAC naïve mice. In PAE females, nuclear MR levels were elevated in both the conditioning only and conditioning plus re-exposure groups relative to the naïve group. Levels of activated ERK2 (phospho-ERK2 expressed relative to total ERK2) protein were elevated in SAC, but not PAE, males following context re-exposure, and a significant interaction between prenatal exposure group and behavioral group was found. No main effects or interactions of behavioral group and prenatal treatment on nuclear ERK2 were found in female mice. These findings suggest a sex difference in which molecular pathways are activated during fear conditioning in mice.

Conclusions: In PAE males, the deficits in contextual fear were associated with the loss of responsiveness of hippocampal formation nuclear GR, MR and ERK2 to signals generated by fear conditioning and context re-exposure. In contrast, the contextual fear deficit in PAE female mice does not appear to be associated with activity-dependent changes in GR and MR levels or ERK2 activation during training or memory recall, although an overall reduction in nuclear GR levels may play a role. These studies add to a growing body of literature demonstrating that, at least partially, different mechanisms underlie learning, memory formation and memory recall in males and females and that these pathways are differentially affected by PAE.

Keywords

fear conditioning; prenatal alcohol; sex difference; glucocorticoid; mineralocorticoid; ERK2; hippocampus

1. BACKGROUND

Studies in both clinical populations and preclinical models have shown that prenatal alcohol exposure (PAE) is associated with impairments in the acquisition, consolidation and recall of learned information, with deficits in hippocampal formation-dependent learning and memory being a common finding.^{1–4} The glucocorticoid receptor (GR), mineralocorticoid receptor (MR) and extracellular signal-regulated kinases 1 and 2 (ERK1, ERK2) are key regulators of hippocampal formation development, structure and functioning^{5–8}, as well as play important roles in various form of learning and memory^{7,9,10}, and, thus, are potential mediators of PAE's effects on this brain region.

Various paradigms, including fear conditioning, have been employed for studying hippocampal formation-dependent learning and memory in rodent models of PAE. Deficits in delay^{11–14} and trace^{13,15,16} fear conditioning have been demonstrated in mouse and rat models of PAE. We previously reported that, compared to adult male offspring of SAC

control dams, adult PAE male mice displayed reduced fear responses, when reintroduced into the training context 24-h following conditioning using a delay (background contextual) conditioning procedure.¹³ In the present studies, we sought to determine the reproducibility of this contextual fear deficit in males and to determine whether adult female PAE mice displayed a similar deficit.

Signaling pathways that are known to play a role in delay fear-conditioned learning, memory formation and memory retrieval in mice include ERK2^{17–21}, NMDA receptors^{22–24}, GRs^{25–27} and MRs^{25,28–31}. We have reported alterations in NMDA receptor-stimulated ERK2 activation³², and GR^{33,34} and MR³³ levels in male PAE mouse hippocampal formation. Therefore, in the present studies, we investigated the effects of acquisition and recall of contextual fear conditioning on GR, MR and ERK2 in male PAE mice and compared these effects with those in female PAE mice, as sex differences have been reported for the effects of PAE on a variety of outcome measures.^{35–47}

Corticosteroids (cortisol in humans and corticosterone in rodents) bind to both the GR and the MR. While GRs are widely distributed throughout the brain, the MR shows a limited distribution, with both receptors enriched in the hippocampal formation.^{10,48–50} GRs and MRs are distributed throughout the cell, being found associated with nuclear, membrane and cytosolic compartments.^{51–55} Current models of GR and MR action propose that nuclear GRs and MRs exert slower onset, genomic actions, whereas plasma membrane-associated GRs and MRs mediate the rapid onset, non-genomic actions of glucocorticoids (GCs).^{55–58} GRs and MRs shuttle between cytosolic and nuclear compartments via both agonist-stimulated⁵⁶ and, at least in the case of the GR, ligand-independent^{59,60} pathways.

Stressful physical and psychological stimuli activate the hypothalamic-anterior pituitary-adrenal (HPA) axis.^{56,61,62} Plasma GC (corticosterone in rodents and cortisol in humans) is used as a measure of HPA activity functioning. Reports on the effects of PAE on basal plasma GC (corticosterone or cortisol) levels and on stressor-induced elevations in plasma GC in preclinical models and clinical populations have provided inconsistent results. Studies have shown that PAE is associated with increased^{39,63–66}, unchanged^{35,44,67–69} or decreased³⁶ basal levels of GC. In a PAE model that allowed mouse dams continuous access to alcohol, we previously reported that basal plasma corticosterone levels measured during the light phase of the light-dark cycle were elevated in female, but not male, offspring.¹¹ Similarly, compared to controls that were not exposed to alcohol prenatally, plasma GC levels measured following stressor experience have been reported to be increased^{35,46,47,64,67,69}, unchanged^{39,70} or decreased^{63,71} in PAE subjects. These findings are influenced by several factors including the age of the subjects, time of day of plasma collection, housing conditions³⁴ and sex of the organism.

We identified similar behavioral deficits in male and female PAE mice but found differing effects on hippocampal formation GR, MR and ERK2. These studies add to a growing body of literature indicating that, at least partially, different mechanisms underlie learning and memory in males and females and that these pathways are differentially affected by PAE.

2. MATERIALS AND METHODS

2.1 Animals

All procedures were approved by the University of New Mexico Health Sciences Center (UNM HSC) Institutional Animal Care and Use Committee and adhered to practices recommended in “Recognition and Alleviation of Pain and Distress in Laboratory Animals” (Committee on Pain and Distress in laboratory Animals, Institute of Laboratory Animal Resources, National Research Council, National Academy Press, Washington D.C. 2011). All of the mice used in this study were offspring of animals bred in the UNM HSC Animal Research Facility and had not experienced any postnatal manipulation other than that occurring during standard animal care prior to the beginning of the study. All animals were maintained on a reverse 12-hour light/12hour dark schedule (lights off at 0800 hours). Adult male and female offspring (126–206 days of age) were used for the present studies. The mice used for the conditioning and 24-h context study (data shown in Figure 1) were from three independent breeding rounds. Tissue from these animals was used for the biochemical analyses (data shown in Figures 2–6). The mice used in the behavioral reproducibility study (data reported in Section 3.1.1) were from a separate (fourth) breeding round. Tissue was not collected from these mice.

2.2 Prenatal alcohol exposure

The PAE paradigm used for these studies has been described previously¹³. Briefly, individually housed C57BL/6J female mice (Jackson Laboratory, Bar Harbor, ME) were provided limited access (4h/d: 10:00 – 14:00) to a solution of either 10% (w/v) ethanol in 0.066% (w/v) saccharin (SAC) or SAC 0.066% (w/v) alone. Stable drinking was established prior to mating, maintained throughout mating and gestation, and withdrawn following parturition using a step-down procedure over a six-day period. Offspring were weaned at 23–25 days of age into group-housed cages of 2–5 same-sex littermates.

Alcohol consumption (4.56 ± 0.16 g ethanol / kg body weight / 4 h, n=25 litters) was similar to that reported in our previous studies^{13,34,72}, in which we found that this level of consumption yields average blood alcohol concentrations of approximately 80–90 mg/dL.

2.3 Synchronization of the female estrous cycle

Inconsistencies have been reported regarding the effect of the estrous cycle phase on fear conditioning. Markus & Zecevic⁷³, using a background contextual conditioning procedure (i.e., a contextual conditioning procedure that includes a conditioned stimulus), reported that rats trained and tested during proestrus showed significantly reduced contextual fear compared to rats trained and tested during estrus and compared to male rats. In contrast, Cossio et al.⁷⁴ reported no effect of estrous cycle phase on context re-exposure freezing in rats trained using either a foreground (i.e., a contextual conditioning procedure that does not include a conditioned stimulus) or a background contextual conditioning procedure. Similarly, Keiser et al.⁷⁵ reported no effect of estrous cycle on foreground contextual conditioning in mice. Our study sought to reduce this potential source of variability by entraining the females into a similar stage of the estrus cycle.

On the day of training (i.e., contextual fear conditioning), mice were in diestrus/proestrus; females experiencing context re-exposure were in proestrus confirmed by visual observation, as described.⁷⁶ Standardization of estrous cycle stage was then confirmed at the time of tissue collection by cytologic evaluation of vaginal lavage samples⁷⁷.

2.4 Delay fear conditioning

Delay fear conditioning and contextual fear memory testing were performed as described.¹³ Training and testing procedures were performed during the dark cycle between 0830 and 1200 hours. All mice to be trained/tested during a session were transported from the vivarium to a room adjacent to the training/testing room. A white noise generator (~70 dB) was placed near the cages. The room was kept dark except as needed to identify cages, which was done under red lighting. Mice were transferred from their home cage to individual cages (standard cages with bedding used for housing mice in the vivarium) and transported to the training/testing room. Both the corridor connecting the holding and training/testing rooms and the training/testing room were illuminated by red light. Mice were placed into the training/testing context within ~30 sec of being transported into the room.

Mice were trained using two pairings of a 30-sec, 80dB, 6Hz clicker (the conditioned stimulus, CS) and a 2-sec, 0.7 mA electric foot shock (the unconditioned stimulus, US) delivered during the last 2 sec of the CS. The intertrial interval (ITI) between the two clicker/shock pairings was 90 sec. Males and females were trained/tested on the same day, with the group of males to be trained/tested being run before the group of females. Immediately after training, mice were transported back to the holding room and returned to their home cage. Thirty to sixty min after completion of the training/testing of the last mouse, mice were transported back to the vivarium. Approximately 24 hours later, subjects were returned to the training context for 3 minutes in order to assess contextual conditioning. Neither the CS nor the US was delivered during this period.

Freezing behavior (absence of movements other than those associated with respiration) occurring during the training and testing periods was scored by two individuals, one of whom was blind to the animal's treatment history. Freezing score differences between the two raters that were greater than 10% were re-scored by a third individual blinded to animal treatment and group condition. Freezing was expressed as a percentage of the total time interval- see text and Figure legends.

Cage mates that did not experience conditioning were used as the "naïve" control group.

2.5 Plasma and tissue collection

Mice were rapidly decapitated without anesthesia. Whole trunk blood was collected in EDTA-treated tubes (Ram Scientific Inc., Yonkers, NY; #077051) and plasma was prepared by immediate centrifugation at room temperature. The brain was removed and the hippocampal formation was isolated. Tissue and plasma samples were snap-frozen in liquid nitrogen and stored at -80°C until use. All tissue collection occurred between 08:30 and 13:00 hours.

In the present studies, we prepared plasma and hippocampal formation subcellular fractions from tissue collected 20 minutes following fear conditioning (conditioning only group) or 20 minutes following re-exposure to the training context (conditioning plus re-exposure group). The choice of times post-behavioral event was based on previous studies in mice demonstrating regulation of ERK2 activation state, as well as changes in mRNA levels of several signaling molecules, in mouse hippocampal formation following fear conditioning or context memory recall.^{20,75,78–83} In addition, plasma and brain tissue were collected from behaviorally naïve (naïve group) mice.

2.6 Plasma corticosterone measurement

Plasma corticosterone concentrations were assessed as described⁸⁴, employing the DetectX Corticosterone EIA Kit (Arbor Assays, Ann Arbor, MI; #K014-H1). Data were analyzed using a Four Parameter Logistic (4PL) curve fit program (Arbor Assays, <https://www.myassays.com/four-parameter-logistic-curve.assay>).

2.7 Isolation and subcellular fractionation of hippocampal formation tissue

Hippocampal formation tissue was briefly thawed and immediately homogenized⁸⁵; nuclear ($1000 \times g_{\max}$, 6 min, 4°C) and membrane ($200,000 \times g_{\max}$, 30 min, 4°C; prepared from the post-nuclear lysate) subcellular fractions were prepared⁸⁶. Quantification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a commonly used marker of cytosolic contamination of subcellular fractions⁸⁷, found that anti-GAPDH immunoreactivity was 14-times more concentrated in the post-nuclear lysate compared to the nuclear fraction (Tyler et al. 2018 in press), indicating that there is minimal contamination of the nuclear fraction with cytosolic components. The fractions were snap-frozen in liquid nitrogen and stored at –80°C until analyzed by immunoblotting.

2.8 Immunoblotting

Immunoblotting was conducted as previously described³³ with the following modifications. GR, MR, ERK1 and ERK2 were assessed in the nuclear fraction using 3 µg total protein and in the membrane fraction using 10 µg total protein. Membranes were blocked for 1 hr in Odyssey Blocking Buffer (LI-COR, Lincoln, NE; product # 927–40100) and incubated overnight in 10 mL PBS with 1% Tween (PBST) with primary antibody directed against GR (1:250 nuclear fraction, 1:100 membrane fraction; Santa Cruz Biotechnology, Dallas, TX; product # sc-376425), MR (1:1000; Santa Cruz product # sc-11412), pErk1/2 (1:2000; Cell Signaling Technology, Danvers, MA; product # 4370), or total Erk1/2 (1:2000; Cell Signaling product # 4696). Unbound antibody was removed with four washes in PBST at room temperature followed by incubation with 15 mL PBST with LI-COR fluorescent antibodies (1:15,000; LI-COR products # 926–68073 and 92632212). Unbound secondary antibody was removed with four washes in PBST and membranes were imaged using a LI-COR Odyssey Infrared Imager (Model 9120). Immunoblotting for pERK1/2 and total ERK1/2 were assessed on the same blot after stripping off GR and MR antibodies with Stripping Buffer (LI-COR product # 928–40028). Immunoreactivity was corrected to their respective Coomassie Stain (10 µg) or Amido Black (3 µg). Amido black proved to be more reliable than Coomassie Stain for lower amounts of total protein.

2.9 Data analysis

In each reported measure, *n* represents a litter- i.e., only one male and one female from a litter were used for each behavioral condition (naïve, conditioning only, conditioning plus context reexposure) or biochemical measure in order to control for litter effects. Data for fear responses during conditioning were analyzed within sex by mixed factor two-way repeated measures, with time interval as the within factor and sex and prenatal treatment as the between groups factors using SPSS (v. 25; IBM). Data for 24 h context fear measures were analyzed by t-test. Data for biochemical studies were analyzed by three-way ANOVAs (between group factors: sex, behavioral group and prenatal treatment) followed by two-way ANOVA within sex (factors: behavioral group and prenatal treatment) using GraphPad Prism (v. 7). Post hoc analyses were run using t-test with Bonferroni correction, as needed. Power calculations (G^* power) for the ANOVA of three behavioral conditions and two prenatal conditions indicate that 85% power is obtained with an effect size ($\eta^2 = 0.2$, $r = 0.45$, $f = 0.55$) at a sample size of $n = 7-10$. For the t-test comparisons, we have 85% power with an achieved Cohen's *d* effect size of 1.4 and an *n* of 9–10.

3. RESULTS

3.1 BEHAVIORAL STUDIES

3.1.1 Both male and female adult PAE mice display deficits in contextual conditioning—Classical conditioning procedures pair a neutral cue, the CS, with a response-evoking stimulus, the US, in a temporally explicit manner, resulting in the formation of an association between the CS and the US. Subsequently, the CS, in the absence of the US, elicits the response, termed a conditioned response. An association also forms between the US and stimuli that are continuously present in the environment in which the US is delivered.^{88–90} Contextual conditioning produced by paradigms that use a phasic CS that is temporally paired with the US is termed background contextual conditioning, while contextual conditioning produced by procedures in which a CS is not presented and the static environmental stimuli serve as the primary predictor of the US is termed foreground contextual conditioning.⁹⁰ In the present studies, we used a background contextual conditioning procedure and assessed contextual fear memory 24 h after training. We chose to use a background, rather than foreground, conditioning procedure based on our focus on the hippocampal formation for the biochemical measures. The hippocampal formation has been shown to play a critical role in background contextual conditioning.^{90–93} In contrast, the hippocampal formation may not be necessary for foreground contextual conditioning⁹⁰, although other studies suggest that it may play a role- for example, see⁷⁹.

When SAC and PAE male and female mice were conditioned as detailed in the Methods section and returned to the training context 24 h later, both male and female PAE mice displayed a deficit in context fear compared to same-sex SAC control mice (Figure 1 top). A two-way ANOVA (factors: sex and prenatal treatment) of the data found no effect of sex, an effect of prenatal treatment [$F(1, 31) = 12.82$, $p = .001$] and no sex by prenatal treatment interaction. Post-hoc analyses showed: SAC male and female mice did not differ in the total level of context fear responding: $t(16) = 0.429$, $p = .674$. PAE vs. SAC males, $t(13) = 2.513$, $p = .026$; PAE females vs SAC females, $t(18) = 2.469$, $p = .024$ (Fig 1 top). However, differences

between males and females were noted when freezing during specific intervals was considered (discussed below, Fig 1 bottom).

We assessed the reproducibility of the contextual fear deficits in a study conducted using mice derived from a breeding round that was separate from the breeding rounds from which the mice for the study shown in Figure 1 top were derived. In this second study, compared to SAC control, both male and female PAE, again, displayed deficits in context fear measured 24-h after conditioning: males, $t(17)=2.184$, $p=.043$; females, $t(17)=3.511$, $p=.003$.

3.1.2 While males and females display dissimilar patterns of fear acquisition, SAC and PAE mice do not differ in fear acquisition—

In order to determine if the observed contextual fear deficit was due to: 1.) an inability of the PAE mice to produce the fear (freezing) response or 2.) a deficit in context fear acquisition during conditioning, we analyzed fear responses during the conditioning session of the mice that displayed the contextual fear deficits shown in Figure 1 top. The data for freezing during the conditioning session are shown in Figure 1 (bottom row).

During training procedures for background contextual fear conditioning, freezing responses occurring following delivery of the US footshock are termed post-shock freezing, and are a conditioned fear response elicited by the training context.^{94,95} Post-shock freezing is a measure of short-term contextual fear memory.⁹⁵ Freezing occurring during the CS (tone) subsequent to the delivery of the first CS-US pairing is a conditioned response to the CS and the context.⁹⁶

The pattern of freezing induced by our delay conditioning procedure in SAC control male mice is similar to that reported by Lattal and colleagues for male C57BL/6J mice.⁹⁷ A mixed design ANOVA with time interval used as the within subjects factor revealed a significant effect of time interval [$F(2.165, 67.1)=226.338$, $p<.0001$], corrected for sphericity using Greenhouse Geisser], and a significant interaction between sex and time interval of freezing [$F(2.165, 67.1)=13.91$, $p<.0001$]. The between groups analysis revealed a significant main effect of sex [$F(21,31)=10.766$, $p=.003$]. Compared to males, females displayed a more abrupt indication of learning with freezing at the 210–240 second time interval and only minimal freezing during the preceding two intervals (90–120 and 120–210). In addition, females did not show a continued increase in freezing during the last interval (240–300 sec) as seen in the males. SAC and PAE mice did not differ in their pattern or level of fear acquisition.

3.2 BIOCHEMICAL STUDIES

3.2.1 Choice of targets and general methods overview—

As noted in the Introduction, GRs^{25–27}, MRs^{25,28–31} and ERK2^{17–21} play a role in fear-conditioned learning, memory formation and memory retrieval in mice; thus, we concentrated our analyses on these molecules. GCs, like other steroid hormones⁹⁸, are thought to enter cells via passive diffusion. Once in the cytosol, GCs can bind to cytosolic GRs or MRs, which mediate the genomic (slower onset and more prolonged) actions of GCs.^{56–58} GCs also act via membrane-associated GRs and MRs, which mediate the non-genomic (relatively rapid onset and short duration) actions of GCs.^{55,58} In order to study the membrane-associated and

nuclear populations of GRs and MRs, we used conventional biochemical methods of differential centrifugation⁸⁶ to isolate membrane and nuclear subcellular fractions from mouse hippocampal formation. Immunoblotting techniques were used to assess GR and MR levels in these fractions.

ERK1 and ERK2 are members of the extracellular signal-regulated kinase family of mitogen-activated protein kinases.⁹⁹ ERK2 plays an important role in fear-conditioned learning and memory^{100,101}, while ERK1 may not^{101,102}. ERK1 and ERK2 reside in both the cytosol and nucleus.^{103–105} We focused our study on the nuclear pool of these molecules, as nuclear ERK1/2 has been implicated in learning and memory.^{106–109} ERK1 and ERK2 catalytic activities are regulated by phosphorylation at two sites near the catalytic site: T183 and Y185 in ERK2.⁹⁹ The activation states of ERK1 and ERK2 are commonly determined using immunoblotting techniques as the ratio of the anti-phospho-ERK1/2 (pERK1/2) immunoreactivity to the anti-total (phosphorylated and unphosphorylated) ERK1/2 immunoreactivity.

3.2.2 PAE altered basal but not fear-induced levels of plasma corticosterone in male and female offspring, resulting in a reduced relative stress response.

—We used plasma corticosterone levels as a measure of the HPA response to conditioning and context re-exposure in SAC and PAE males and females (Figure 2 top row). A three-way ANOVA revealed a significant overall effect of sex [$F(1,66) = 36.229$, $p < .001$], an effect of behavioral group [$F(2,66) = 64.63$, $p < .0001$] and a sex by behavioral group interaction [$F(2,66) = 9.911$, $p < .0001$]. To probe the source of the sex difference in plasma corticosterone concentrations, separate 2-way ANOVAs were used to analyze the male and female data sets. For both sexes, there was a significant effect of behavioral group (male: [$F(2, 28) = 23.84$, $p < .0001$]; female: [$F(2, 36) = 47.1$, $p < .0001$]) and no effect of prenatal treatment or interaction between these factors. Comparisons of basal corticosterone concentrations demonstrated that these concentrations were elevated in male ($t(10) = 2.846$, $p = .017$) and female ($t(12) = 2.912$, $p = .013$) PAE mice relative to their sex-matched SAC controls.

For this study, litter mates from the same cage were used, allowing us to express plasma corticosterone concentrations measured in conditioning only and conditioning plus re-exposure animals relative to their behaviorally naïve cage mates (Figure 2 bottom row). Two-way ANOVAs of the data sets reported a significant effect of prenatal treatment (male: [$F(1, 18) = 14.68$, $p = .001$]; female: [$F(1, 24) = 16.75$, $p < .001$]), but no effect of behavioral group or interaction of these two factors. Post hoc analyses revealed that, relative to naïve values, the elevations of plasma corticosterone concentrations that were associated with conditioning only and conditioning plus re-exposure were smaller in PAE mice than in SAC controls: male conditioning only, $t(8) = 2.301$, $p = .050$; male conditioning plus re-exposure, $t(10) = 3.195$, $p = .010$; female conditioning only, $t(12) = 2.743$, $p = .018$; female conditioning plus re-exposure, $t(12) = 3.053$, $p = .010$.

3.2.3 PAE exerts sex-specific effects on membrane GR and MR levels

3.2.3.1 Membrane GR and MR levels are elevated in naïve PAE males but not females; context re-exposure induced a decrease in membrane MR levels in PAE males:

We detected an anti-GR immunoreactive band having an apparent molecular weight of 95 kDa in membrane fractions prepared from mouse hippocampal formation. Three-way ANOVA revealed a significant sex effect [$F(1,96) = 8.665, p = .004$] and a significant interaction between sex and prenatal condition [$F(1, 96) = 4.493, p = .037$]. A two-way ANOVA revealed a significant effect of prenatal treatment on membrane-associated GR levels [$F(1, 40) = 4.274, p = .045$] but no overall effect of behavioral group or interaction between prenatal treatment and behavioral group in males (Figure 3 top left). Post hoc analysis revealed that PAE was associated with increased basal levels of membrane-associated GR in males ($t(15) = 3.309, p = .005$). There were no significant overall effects of prenatal treatment or behavioral group, or interaction between these factors, in females (Figure 3 top right).

We detected an anti-MR immunoreactive band having an apparent molecular weight of 102 kDa in hippocampal formation membrane fractions. There was a significant effect of sex on membrane levels of MR [$F(1,72) = 292.66, p < .0001$]. In males, two-way ANOVA showed that there was a significant effect of prenatal treatment on membrane-associated MR levels [$F(1, 28) = 4.405, p = .045$] but no overall effect of behavioral group or interaction between prenatal treatment and behavioral group (Figure 3 bottom left). A post hoc t-test showed that PAE was associated with increased basal levels of membrane-associated MR in males ($t(10) = 2.819, p = .018$).

Based on a study of Zhou et al.²⁹ showing that the MR plays an important role in context fear memory recall, an effect that may be mediated by membrane-associated MRs¹¹⁰, we performed t-tests comparing data for the conditioning plus re-exposure groups to naïve controls. Membrane MR levels were significantly reduced in PAE conditioning plus re-exposure vs PAE naïve mice ($t(10) = 2.301, p = .044$); SAC conditioning plus re-exposure and SAC naïve levels were not different from each other.

There were no significant overall effects of prenatal treatment or behavioral group, or interaction between these factors, when the data for females were analyzed (Figure 3 bottom right).

3.2.4 Fear conditioning and PAE exert sex-specific effects on nuclear GR and MR levels and ERK2 activation state

3.2.4.1 SAC but not PAE males displayed increased nuclear GR and MR levels following fear conditioning and context re-exposure, respectively; PAE reduced nuclear GR levels, as well as elevated nuclear MR levels following conditioning and context re-exposure, in females.:

We detected primarily two anti-GR immunoreactive proteins in nuclear fractions prepared from male and female hippocampal formation: one of apparent molecular weight of 95 kDa and one of 90 kDa. In males and females, no significant effects of prenatal treatment, behavioral group, or interaction between these two factors, was seen in

two-way ANOVA analyses of data for the 95 kDa band. Therefore, the following discussion is based on the analyses of the 90 kDa form of the nuclear GR.

Figure 4 (top row) shows the data for nuclear GR levels measured in naïve SAC control and PAE male (Left) and female (Right) mice, as well as in nuclear fractions prepared from tissue collected 20 minutes following conditioning and 20 minutes following context re-exposure. A three-way ANOVA revealed a significant interaction between sex, prenatal condition and behavioral group [$F(2,95) = 5.49, p=.006$], as well as a sex by behavioral group interaction [$F(2, 95) = 10.067, p<.0001$], a main effect of sex [$F(1,95) = 33.517, p<.0001$] and a main effect of prenatal treatment condition [$F(1,95) = 12.888, p=.001$]. In males, a two-way ANOVA showed that there was a significant interaction between prenatal treatment and behavioral group [$F(2, 35) = 4.298, p=.021$], a significant overall effect of behavioral group [$F(2, 35) = 5.124, p=.011$] and an effect of prenatal treatment that approached significance [$F(1, 35) = 3.333, p=.076$]. Post hoc analyses using t-tests found significant differences between: SAC conditioning only and SAC naïve ($t(12)=3.203, p=.008$), SAC conditioning only and PAE conditioning only ($t(11)=3.091, p=.010$) and SAC conditioning only and SAC conditioning plus re-exposure ($t(10)=3.541, p=.005$).

In females, there was a significant effect of prenatal treatment on nuclear GR levels [$F(1, 34) = 5.28, p=.028$] but there was no effect of conditioning group or interaction. Within a conditioning group (naïve, conditioning only, conditioning plus context re-exposure), post hoc t-tests found that none of the SAC vs. PAE differences were significant.

In nuclear fractions, we detected primarily two anti-MR immunoreactive bands: one of 127 kDa and one of 133 kDa. Attempts to analyze these bands separately were unreliable; therefore, the following discussion is based on quantification of the immunoblot region encompassing both bands.

Figure 4 (bottom row) shows data for MR levels in hippocampal formation nuclear fractions isolated from the three behavioral groups of SAC control and PAE male (Left) and female (Right) mice. There was a significant effect of sex on nuclear MR [$F(1,95) = 4.759, p=.32$], as well as a main effect of behavioral group [$F(2,95) = 19.686, p<.0001$]. In males, we found a significant effect of behavioral group [$F(2, 31) = 4.06, p=.027$] but no effect of prenatal exposure or interactions between these two factors. A post hoc t-test found that nuclear MR levels were significantly elevated in SAC conditioning plus re-exposure mice compared to SAC naïve mice ($t(11)=3.101, p=.010$).

In females, we also found a significant effect of behavioral group [$F(2, 37) = 5.333, p=.009$] and no effect of prenatal exposure or interaction between behavioral group and prenatal exposure. Within a prenatal exposure groups, t-test analyses found that, compared PAE naïve mice, PAE conditioning only ($t(10)=2.675, p=.023$) and PAE conditioning plus re-exposure ($t(11)=2.239, p=.047$) mice had significantly elevated nuclear MR levels.

3.2.4.2 Context re-exposure is associated with ERK2 activation in SAC control but not PAE males; fear conditioning, context re-exposure and PAE did not alter ERK1/2 levels or activation state in female mice.: We assessed levels of pERK1, pERK2, total

ERK1 and total ERK2 in male (Figure 5) and female (Figure 6) SAC and PAE mice in the three behavioral groups. In addition, we calculated the activation states of ERK1 and ERK2 as the ratio of phospho-to-total forms of the enzymes: i.e., anti-pERK1 immunoreactivity / anti-total ERK1 immunoreactivity and anti-pERK2 immunoreactivity / anti-total ERK2 immunoreactivity.

Sex differences in pERK1, pERK2, total ERK1, total ERK2 and the pERK/total ratios were assessed using three way ANOVAs. For pERK 1, there was a main effect of sex on pERK1 levels [$F(1,94)=774.93$, $p<.0001$] and a sex effect of ERK1 total [$F(1,95)=740.03$, $p<.0001$], as well as a main sex effect in pERK1/total ratio [$F(1,95)=188.73$, $p<.0001$]. For sex assessments on pERK2, there was a significant main sex effect [$F(1,95)=841.437$, $p<.0001$] and a significant interaction between sex and behavioral group [$F(2,95)= 3.439$, $p=.036$]. Total ERK2 showed an effect of sex [$F(1,95)=1050.22$, $p<.0001$] and a sex by behavioral group interaction [$F(2,95)=3.992$, $p=.022$]. Analysis of the ratio of pERK2/ total ERK2 revealed a significant main effect of sex [$F(1,95)= 47.45$, $p<.0001$], a sex by behavioral group interaction [$F(2,95)=9.242$, $p<.0001$] and a prenatal treatment by sex by behavioral group interaction [$F(2,95)=13.479$, $p<.0001$]. These analyses support the observation that behavior and prenatal treatment changes tended to occur in males and not in females (Figures 5 and 6).

In males (Figure 5), two-way ANOVA analysis of anti-pERK1 immunoblotting data revealed that there was not a significant effect of prenatal treatment but a significant effect of behavioral group [$F(2, 34) = 4.843$, $p=.014$] and a significant interaction between prenatal exposure and behavioral group [$F(2, 34) = 4.452$, $p=.019$]. Post hoc analysis of the data revealed that pERK1 levels in the SAC conditioning plus context re-exposure animals were significantly higher than the levels in the SAC naïve mice ($t(11)=3.645$, $p=.004$) and there was a significant difference between SAC and PAE conditioning plus re-exposure groups ($t(10)=2.767$, $p=.020$). There was no effect of prenatal exposure, behavioral group, or interaction between these factors on total ERK1 in males. Similarly, analysis of the ERK1 activation (pERK1 / total ERK1) data showed no effect of prenatal exposure, behavioral group or interaction between these factors.

Two-way ANOVA analysis of the pERK2 data in males found that there was not a significant effect of prenatal exposure but there was a significant effect of behavioral group [$F(2, 34) = 4.644$, $p=.016$] and a significant interaction between behavioral group and prenatal treatment [$F(2, 34) = 8.053$, $p=.001$]. Post hoc analyses using t-tests revealed significant differences between the SAC naïve and SAC conditioning plus re-exposure groups ($t(11)=4.646$, $p=.0007$), the SAC conditioning only and SAC conditioning plus re-exposure groups ($t(10)=2.935$, $p=.015$) and the SAC conditioning plus re-exposure and PAE conditioning plus re-exposure groups ($t(10)=4.327$, $p=.002$). There was a significant effect of behavioral group [$F(2, 34) = 3.635$, $p=.037$] but not of prenatal exposure or interaction between these factors found when the total ERK2 data were analyzed. Post hoc t-tests found that the only significant difference between behavioral groups was for the SAC naïve vs SAC conditioning plus re-exposure ($t(11)=2.887$, $p=.015$). Analyses of the pERK2 / total ERK2 data revealed no effect of prenatal exposure or of behavioral group but a significant interaction between these factors [$F(2, 34) = 7.496$, $p=.002$]. Post hoc t-tests found

significant differences between SAC naïve and SAC conditioning plus re-exposure groups ($t(11)=2.422$, $p=.034$), SAC and PAE conditioning plus re-exposure groups ($t(10)=2.908$, $p=.016$), PAE naïve and PAE conditioning plus re-exposure ($t(12)=2.758$, $p=.017$) and PAE conditioning only vs PAE conditioning plus re-exposure ($t(11)=2.773$, $p=.018$). Interestingly, after context re-exposure, ERK2 activation was significantly increased in SAC mice and decreased in PAE mice compared to SAC and PAE naïve mice, respectively.

In females (Figure 6), analyses of both the ERK1 and ERK2 data revealed no significant effects of prenatal exposure, behavioral group or interaction of these factors for pERK1, total ERK1, the pERK1/total ERK1 ratio, pERK2, total ERK or the pERK2/total ERK2 ratio. The effect of conditioning did approach significance [$F(2, 39) = 2.738$, $p=.077$] for total ERK2.

4. DISCUSSION

There are two lines of findings from these studies. The first is that adult male and female SAC control mice trained with a background contextual conditioning procedure displayed similar levels of contextual fear memory recall but markedly different profiles of biochemical changes associated with training and context re-exposure. The second line of findings relates to the presence of deficits in contextual fear in PAE males and females and the biochemical effects of PAE that may underlie these deficits.

We found no difference between male and female control (SAC) mice in background contextual conditioning. This is in agreement with a report by Sweatt and colleagues.¹¹¹ However, some studies have reported that males exhibit greater background contextual conditioning than do females.^{112–116} It is likely that this apparent inconsistency between studies is due to differences in training parameters (e.g., the strength of the US^{112,114}), housing (isolation vs group), light cycle phase or, possibly, the estrous stage of females⁷³. Similarly, while some studies show that males display greater foreground contextual conditioning than do females^{82,112,117,118}, other studies report no sex difference^{118–121} or greater conditioning in females⁷⁵. Interestingly, we did find a sex difference in the pattern of fear acquisition in males and females.

While PAE males and females displayed a reproducible deficit in contextual fear memory, the data in Figure 1 (bottom) show that, within a sex, the conditioning procedure elicited similar levels and patterns of fear responding in SAC and PAE mice. The lack of an effect of PAE on post-shock freezing indicates that short-term contextual fear memory⁹⁵ is not disrupted in the PAE mice. Further, the lack of a difference in freezing occurring during the second CS (tone) shows that PAE mice do not differ from SAC controls in their conditioned response to the CS and the context⁹⁶ during the training session. These data demonstrate that SAC and PAE mice do not differ in fear acquisition or in their ability to produce the freezing response.

The lack of a difference in fear acquisition indicates that the observed contextual conditioning deficit observed in the 24-h context re-exposure test in PAE males and females is due to an impairment in consolidation and/or recall of the contextual fear memory. It is of

note that deficits in contextual fear memory in animals displaying normal fear responding during training have been reported in studies in which GR antagonist was administered either peripherally or centrally into the lateral ventricle, ventral hippocampus, or basolateral amygdala prior to contextual conditioning.¹²²

Basal plasma corticosterone levels were elevated in male and female PAE mice relative to SAC control mice. In contrast, corticosterone levels measured following fear conditioning and following memory recall were similar in SAC and PAE mice. When the plasma corticosterone levels in the conditioning only and conditioning plus context re-exposure mice were expressed relative to naïve controls, the relative increases calculated for the PAE mice were smaller than those calculated for the same sex SAC mice. In several biologic systems, including cell signaling, response amplitude and duration are determined by the relative, rather than absolute, change produced by an input signal.^{123–125} Thus, the reduced fold-change in corticosterone levels following conditioning and fear memory recall in PAE mice is predicted to result in reduced regulation of downstream signaling responses in these animals. In males, this is what was observed for the regulation of nuclear GR levels following training and nuclear MR levels and ERK2 activation following context re-exposure. In addition to the effects associated with PAE, we found a sex difference in the corticosterone response produced by the fear conditioning and context re-exposure procedures. While conditioning and context re-exposure were associated with elevations in plasma corticosterone in males and females, the magnitude of the increases relative to baseline levels were greater in females than in males.

We found that, 20 min following training, hippocampal formation nuclear GR levels were increased in SAC males but not PAE males, SAC females or PAE females. We are unaware of any studies that have shown that fear conditioning is associated with an increase in nuclear GR levels. However, several studies have shown that hippocampal formation nuclear GR levels are increased following other forms of stress, including restraint stress^{126–133}, elevated plus maze experience^{134,135}, social defeat stress¹³⁶, forced swim¹³⁷ and predator odor exposure¹³⁸.

Several studies have implicated the GR as being important for the consolidation, but not acquisition, of fear memory. Pre-training administration of GR antagonist blocked context fear in rats trained using a foreground^{122,139–141} or background^{25,141} contextual conditioning procedure. Similarly, knockdown of GRs in the inner layer of the dentate gyrus of the hippocampal formation, preferentially in newborn neurons, blocked context fear memory produced by a background contextual conditioning procedure in mice.²⁷ These studies, combined with the observation that nuclear levels of GR are increased by several stressors, indicate that the lack of an elevation in nuclear GR levels in PAE mice 20 min post-training is a potential contributing mechanism to reduced consolidation of context fear memory in these animals and, thus, the observed reduced context fear response upon context re-exposure.

Impaired GC-induced nuclear translocation of the GR, as well a variety of other mechanisms, have been reported to reduce cellular responsiveness to the actions of GCs.^{142–148} GC resistance (i.e., reduced sensitivity to the actions of GCs) is associated with a

variety of chronic diseases¹⁴⁹, several of which are also associated with PAE: asthma¹⁵⁰, metabolic syndrome¹⁵¹, insulin resistance^{152–155}, depression^{156–158}, inflammatory and disorders^{159–163}. Thus, our data identify reduced activity-dependent nuclear translocation of the GR as a mechanism that may contribute to, at least some forms of, GC resistance associated with PAE.

Context re-exposure increased nuclear MR levels in SAC males, but not PAE males or SAC females. This increase either may be an important part of the recall process in control males or may be a consequence of recall and, therefore, is likely to play a role in memory reconsolidation. A study by Zhou et al.²⁹ shows that administration of spironolactone, an MR antagonist, shortly prior to context re-exposure impaired context memory recall on the day of administration but exerts no effect on freezing in subsequent context tests. This indicates that the MR plays a critical role in the retrieval, but not the reconsolidation, of contextual fear memory. Thus, the increase in nuclear MR levels that was recorded 20 min after context re-exposure in the SAC males and was not present in the PAE males suggests that impaired regulation of nuclear MR levels may be an important mechanism contributing to the context fear recall deficit in the PAE males.

Dorey et al.¹¹⁰ reported that membrane MRs play an important role in memory recall in male mice. While our data do not support a role for membrane MRs in the recall of context fear memory in SAC males, we did find that membrane MR levels were reduced in PAE males following context re-exposure compared to PAE naïve males. It is possible that this decrease contributes to the deficit in context fear observed in the PAE males. In SAC females, we found no effect of contextual conditioning or context memory recall on membrane or nuclear MRs. We did find, however, that nuclear MR levels were increased both after conditioning and after context re-exposure in PAE females compared to PAE naïve females. This dysregulation could contribute to the deficit in PAE females or could be an attempted compensatory mechanism in these animals. The latter possibility is less likely than the former, as overexpression of the MR has been reported to not affect context fear in female mice.¹⁶⁴

It is noteworthy that we found a prenatal treatment by sex by behavioral group interaction for the ratio of pERK2 / total ERK2. Consistent with the existing literature^{19,21,83,165,166}, context re-exposure was associated with hippocampal formation ERK2 activation in SAC males. In contrast, there was no effect of context re-exposure on ERK2 activation in PAE males, SAC females or PAE females. Hippocampal formation ERK1/2 activation has been shown to play a role in retrieval^{19,83,166,167} and extinction^{165,167}, but not reconsolidation^{19,165,167}, of contextual fear memory. Intra-hippocampal infusion¹⁹ or intraperitoneal injection¹⁶⁷ of an inhibitor of mitogen activated protein kinase kinase (MEK), the kinase responsible for the phosphorylation of ERK1/2, 15 minutes¹⁹ or 1 hour¹⁶⁷ prior to context re-exposure has been shown to reduce fear memory retrieval. The magnitude of hippocampal formation pERK1/2 activation measured following context re-exposure is correlated with the strength (number of US stimuli) of the training procedure⁸³ and the amount of freezing measured in the context test^{83,166}. These findings support the conclusion that the reduction in post-retrieval ERK2 activation that we observed in the PAE

males plays an important role in the observed context memory deficit exhibited by these animals.

The anti-total ERK1/2 antibody used in these studies was raised against a portion of the ERK1 and ERK2 proteins that varies in amino acid sequence. Although it is a monoclonal antibody, it may have different affinities for similar, but slightly different, epitopes in the two proteins and, thus, we are unable to compare total ERK1 and total ERK2 in our samples. The monoclonal anti-phospho-ERK1/2 antibody that we used does recognize the same epitope in both proteins and, therefore, we were able to compare the pERK1:pERK2 ratio in our mice. In SAC males and SAC females, ratios of approximately 1:6 for pERK1:ERK2 in hippocampal formation nuclear fractions were detected. This finding is similar to a report by Lefloch et al.¹⁶⁸, who found that ERK1:ERK2 and pERK1:pERK2 ratios vary from 1:2 to 1:13 across mouse brain regions. As pERK1 and pERK2 have similar catalytic activities^{168,169} and it appears that the kinases phosphorylate the same set of proteins within a cell¹⁷⁰, the 5-fold higher levels of pERK2 in hippocampal formation indicate that it is likely to play a more important role than pERK1 in the retrieval of contextual fear memory.

We have previously reported a deficit in NMDA receptor-dependent ERK1/2 activation in the dentate gyrus of male mice exposed to alcohol prenatally³². The PAE model used in those studies allowed dams continuous access to ethanol throughout pregnancy, whereas in the present studies we used a limited access (drinking-in-the-dark) ethanol paradigm.¹³ Additionally, the tissue used in the previous study was collected during the light phase of the light cycle, while in the present studies the behavior and tissue collection occurred during the dark phase of the light cycle. Thus, although there are methodological differences between our previous and present studies, the findings agree. Lindquist and colleagues¹⁷¹ reported antipERK1/2 immunoreactivity was reduced in the hippocampal formation of rats exposed to ethanol on postnatal day 4 to 9. ERK1/2 has also been identified a key component of networks that are regulated by acute¹⁷² and chronic¹⁷³ alcohol consumption in adult mice. Thus, ERK1/2 appears to be a key target of alcohol actions.

The lack of effects of conditioning on nuclear GR levels and of fear memory recall on nuclear MR levels and ERK2 activation in SAC females support the contention that males and females employ different mechanisms for learning and memory during contextual conditioning and context fear memory recall. Several published studies demonstrate that fear conditioning is associated with relatively rapid changes in the levels / activation state of various signaling molecules in the hippocampal formation of males but not females following training^{81,113,114,174} and following context reexposure⁷⁵. It should be noted, however, that there are reports showing that, for some effectors, fear conditioning is associated with similar changes in males and females⁷⁵ or greater elevations in females compared to than males.⁸² An alternative explanation for our data is that males and females use the same mechanisms for learning, memory and memory recall but have differing kinetics for regulation of the signaling pathways. We cannot rule out this latter possibility, as our studies captured information at a single time after training and after memory recall. However, in the case of context memory recall, Keiser et al.⁷⁵ concluded that, while males primarily rely on hippocampal formation pathways, females appear to shift toward amygdalar processing during retrieval of context fear-associated memories. Finally,

analogous to our findings, Homiack et al.¹⁷⁵ reported that 2,5-dihydro-2,4,5-trimethylthiazoline, a component of fox urine, produced similar behavioral responses (e.g. increased avoidance behavior and increased immobility) in male and female Wistar rats but differing neurochemical effects (changes phosphorylation of cyclic-adenosine monophosphate response element binding protein and Erk1/2) in the hippocampal formation.

5. CONCLUSIONS

While male and female SAC control mice displayed similar levels of freezing in the context fear recall test, we found several differences in the effects of training and context re-exposure on biochemical measures in SAC males and females. Our data and the existing literature show that, in control males, nuclear GR levels increase following training, while context re-exposure is associated with an increase in nuclear MR levels and activation of nuclear ERK2. In contrast, these biochemical changes either are not associated with training and context re-exposure in control females or occur on a different time scale than in males. Thus, although both males and females activate the hippocampus during acquisition of fear conditioning^{75,82}, our results and the findings of other investigators^{75,81,82,111,113,114,120,174,176} indicate that there are sex differences in the molecular mechanisms that underlie the acquisition, consolidation and retrieval of fear conditioning.^{17, 18}

Both male and female PAE mice displayed deficits in context fear. In PAE males, the deficits in context fear were associated with impaired regulation of signaling pathways associated with fear memory consolidation (nuclear GR levels) and fear memory recall (nuclear MR levels and ERK2 activation). Other mechanisms (e.g., a reduction in fear memory recall-associated membrane levels of MR) are likely to contribute to the context fear deficit in PAE males. In contrast, we were unable to identify effects of training or context memory recall on hippocampal formation GR and MR levels or ERK2 activation that may underlie the context learning/memory deficit in PAE female mice; however, it is possible that an overall reduction in nuclear GR levels plays a role.

In conclusion, these studies add to a growing body of literature indicating that, at least partially, different mechanisms underlie acquisition, consolidation and recall in males and females.

Further, the studies show that these pathways are differentially affected by PAE in a sex-specific manner.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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HIGHLIGHTS

- Fear conditioning induced biochemical changes in a sex-dependent manner
- Male and female prenatal alcohol exposure (PAE) mice displayed context deficits
- PAE was associated with loss of regulation of several nuclear signalss

Figure 1.

Top: Prenatal alcohol exposure (PAE) was associated with reduced contextual fear in adult male and female mice. Saccharin (SAC) control and PAE offspring were trained with a background contextual conditioning procedure. 24-hr later, mice were returned to the training context and fear responses (freezing) were measured. In the figure, the data are expressed as a percentage of time spent freezing during the 3-minute context test. Data are presented as mean + S.E.M. Males: SAC n=9, PAE n=10; females: SAC n=10, PAE=9. *, $p < .05$; **, $p < .01$. **Bottom:** SAC control and PAE male (left) and female (right) offspring displayed differences in percent freezing depending on the time interval during the conditioning session. Fear responses (freezing) were assessed prior to the first tone-shock pairing (0–90 sec), during the first tone-shock presentation (90–120 sec), during the ITI, post-shock period (120–210 sec), during the second tone-shock pairing (210–240) and during the second post-shock period (240–300 sec). In the figure, the data are expressed as a percentage of time spent freezing during the specified time intervals. Data are presented as mean $\pm$ S.E.M. Males, SAC n=8, PAE n=7; females SAC n=10, PAE n=10.

Figure 2.

Although plasma corticosterone concentrations measured following contextual fear conditioning (conditioning only, CO) and context re-exposure (conditioning plus re-exposure, C+R) were similar in saccharin (SAC) control and prenatal alcohol exposure (PAE) male (left) and female (right) mice (top row), when corticosterone levels were normalized to behaviorally naive cage-mates (bottom row), the CO- and C+R-dependent increases in corticosterone levels were reduced in PAE male and female mice. **Top row:** Corticosterone levels were measured in plasma prepared from blood collected from naive, CO and C+R mice, as detailed in the Methods. Male: SAC and PAE, naive n=6, CO n=5, CO +R n=6. Female: n=7 for all groups. **Bottom row:** The data for CO and C+R mice were normalized to their same-sex cage mate who was behaviorally naive and expressed as fold-change. Data are presented as mean + S.E.M. *, p<.05; **, p<.01.

Figure 3.

*Prenatal alcohol exposure (PAE) was associated with increased basal levels of membrane-associated glucocorticoid receptor (GR; top row) and mineralocorticoid receptor (MR, bottom row) in male (left column) but not female (right column) mice. Male PAE mice displayed a reduction in membrane MR following conditioning plus context re-exposure. Anti-GR and anti-MR immunoreactivities were measured in the membrane fraction prepared from behaviorally naïve (naïve), contextual conditioning (conditioning only, CO) and contextual conditioning plus context re-exposure (conditioning plus re-exposure, C+R) male and female saccharin (SAC) control and PAE mouse hippocampal formation. The number of mice per behavioral treatment was: Male SAC: n=7–8 for GR and n=5–6 for MR, Male PAE: n=7–9 for GR and n=6 for MR; Female SAC: n=8–9 for GR and n=5–6 for MR, Female PAE: n=5–8 for GR and n=5–7 for MR. Data are presented as mean ± S.E.M. *, p < 0.05; **, p < 0.01*

Figure 4.

*Nuclear glucocorticoid receptor (GR; top row) and mineralocorticoid receptor (MR; bottom row) levels were elevated following fear conditioning and context re-exposure, respectively, in hippocampal formation of SAC but not PAE males (left column). In female (right column) mouse hippocampal formation, PAE reduced nuclear GR levels, as well as elevated nuclear MR levels following conditioning and context re-exposure. Anti-GR and anti-MR immunoreactivities were measured in the nuclear fraction prepared from behaviorally naive (naive), contextual conditioning (conditioning only, CO) and contextual conditioning plus context re-exposure (conditioning plus re-exposure, C+R) male and female saccharin (SAC) control and PAE mouse hippocampal formation. The number of mice per behavioral treatment was: Male SAC: n=6–8 for GR and n=6–7 for MR, Male PAE: n=6–8 for GR and n=5–7 for MR; Female SAC: n=7 for GR and n=8–9 for MR, Female PAE: n=5–7 for GR and n=6–7 for MR. Data are presented as mean $\pm$ S.E.M. *, p<0.05; **, p<0.01.*

Figure 5.

Context re-exposure was associated with ERK2 activation in saccharin (SAC) control, but not prenatal alcohol exposure (PAE) males. Anti-phospho-extracellular signal-regulated kinase 1 (pERK1) (left) and anti-pERK2 (right) immunoreactivities (**top row**), anti-total ERK1 (left) and anti-total ERK2 (right) immunoreactivities (**middle row**) and the calculated anti-pERK1 / anti-total ERK1 (left) and anti-pERK2 / anti-total ERK2 (right) ratios (**bottom row**) in the nuclear fraction prepared from behaviorally naive (naive), contextual conditioning (conditioning only, CO) and contextual conditioning plus context re-exposure (conditioning plus re-exposure, C+R) male SAC and PAE mouse hippocampal formation.

SAC n =6–7 per behavioral group and PAE n=6–8 per behavioral group. Data are presented as mean $\pm$ S.E.M. *, $p<.05$; **, $p<.01$.

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Figure 6.

Lack of effects of fear conditioning, context re-exposure and prenatal treatment on ERK1 and ERK2 activation in female mice. Anti-phospho-extracellular signal-regulated kinase 1 (pERK1) (left) and anti-pERK2 (right) immunoreactivities (**top row**), anti-total ERK1 (left) and anti-total ERK2 (right) immunoreactivities (**middle row**) and the calculated anti-pERK1 / anti-total ERK1 (left) and anti-pERK2 / anti-total ERK2 (right) ratios (**bottom row**) in the nuclear fraction prepared from behaviorally naive (naive), contextual conditioning (conditioning only, CO) and contextual conditioning plus context re-exposure (conditioning plus re-exposure, C+R) female SAC control and PAE mouse hippocampal

formation. Data are presented as mean $\pm$ S.E.M. SAC n =8–9 per behavioral group and PAE n=5–8 per behavioral group.

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