YALE JOURNAL OF BIOLOGY AND MEDICINE 95 (2022), pp.87-104.

ORIGINAL CONTRIBUTION

YALE JOURNAL OF BIOLOGY AND MEDICINE
YJBM
CELEBRATING 90 YEARS 1928–2018

# The Impact of Maternal Antioxidants on Prenatal Stress Effects on Offspring Neurobiology and Behavior

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Prenatal stress is a neuropsychiatric risk factor, and effects may be mediated by prenatal oxidative stress. Cell types in the brain sensitive to oxidative stress—cortical microglia and cortical and hippocampal interneurons—may be altered by oxidative stress generated during prenatal stress and may be neurobiological substrates for altered behavior. Our objective was to determine the critical nature of oxidative stress in prenatal stress effects by manipulating prenatal antioxidants. CD1 mouse dams underwent restraint embryonic day 12 to 18 three times daily or no stress and received intraperitoneal injections before each stress period of vehicle, N-acetylcysteine (200 mg/kg daily), or astaxanthin (30 mg/kg before first daily stress, 10 mg/kg before second/third stresses). Adult male and female offspring behavior, microglia, and interneurons were assessed. Results supported the hypothesis that prenatal stress-induced oxidative stress affects microglia; microglia ramification increased after prenatal stress, and both antioxidants prevented these effects. In addition, N-acetylcysteine or astaxanthin was effective in preventing distinct male and female interneuron changes; decreased female medial frontal cortical parvalbumin interneurons was prevented by either antioxidant; increased male medial frontal cortical parvalbumin interneurons was prevented by N-acetylcysteine and decreased male hippocampal GAD67GFP+ cells prevented by astaxanthin. Prenatal stress-induced increased anxiety-like behavior and decreased sociability were not prevented by prenatal antioxidants. Sensorimotor gating deficits in males was partially prevented by prenatal astaxanthin. This study demonstrates the importance of oxidative stress for persistent impacts on offspring cortical microglia and interneurons, but did not link these changes with anxiety-like, social, and sensorimotor gating behaviors.

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Abbreviations: PBS, phosphate buffered saline; NAC, n-acetylcystine; AST, astaxanthin; NS, non-stressed; PS, prenatally-stressed; mFC, medial frontal cortex; PV, parvalbumin; GAD67, Glutamic acid decarboxylase 67; GFP, green fluorescent protein; EPM, elevated plus maze; OF, open field; ANOVA, analysis of variance; dB, decibels; PPI, prepulse inhibition.

Keywords: prenatal stress, antioxidants, microglia, interneuron, behavior, mouse

Author Contributions: JL-BD: Conception, design, experiments, data collection and analysis, writing, funding; MO: writing, review, editing; HE: data collection, funding; SLS: data collection, funding; HES: Conception, design, data analysis, writing, review, editing, funding.

## INTRODUCTION

Prenatal stress is associated with an increased risk for neuropsychiatric illness including attention-deficit hyperactivity disorder, autism spectrum disorder (ASD), and schizophrenia [1-4], as well as increasing childhood behavioral, physiological, and emotional problems in offspring [5-7]. Specifically, prenatal stress is associated with disruptive behavioral problems in toddlers, increased risk for lower motor function in kindergartners, and more externalizing behaviors in older children [8-10]. Alterations to the developing brain during prenatal stress must have enduring consequences to explain the development of neuropsychiatric illness many years later [11].

Prenatal stress may have persistent effects on brain function through redox dysregulation that occurs during stress [12-15]. Embryonic brain is vulnerable to changes in reactive oxygen species and redox dysregulation because of its low antioxidant capacity and other characteristics [16-20]. Prenatal stress increases oxidative stress in embryonic brain [21]. Maternal treatment during pregnancy with N-acetyl cysteine (NAC), a glutathione precursor and well-established antioxidant, prevents learning and memory deficits brain redox changes in adult offspring exposed to prenatal stress or maternal inflammation [22-25]. These findings suggest that neurobiological substrates sensitive to oxidative stress may underlie prenatal stress effects on additional domains of behavior relevant to neuropsychiatric disorders.

Microglia are critical regulators of a range of brain functions during development and in mature brain [26-30], with abnormalities of microglia linked with neuropsychiatric disorders [31-33]. More microglia with reduced or thicker ramifications in cortical regions of individuals with neuropsychiatric disorders have been hypothesized to be linked to the role of these activated or "primed" cell types in pathophysiological synaptic pruning [33,34]; a range of behaviors may be affected by mis-targeted synaptic pruning by activated or primed microglia in developing and/or mature brain. Microglia are reactive to oxidative stress [35-37] and are altered by prenatal stress [38-41], further implicating oxidative stress in persistent outcomes of prenatal stress.

GABAergic cortical interneurons are also affected by prenatal stress [42-45] and are implicated in the neuropsychiatric deficits linked to prenatal stress [46-49]. Cortical and hippocampal GABAergic populations contribute to excitatory/inhibitory balance and oscillations within circuits, which coordinate neuronal firing necessary for behavioral regulation and are hypothesized to have pathophysiological roles in ASD, schizophrenia, and other neuropsychiatric disorders [50]. The appropriate proportion of interneurons expressing parvalbumin and capable of fast-spiking activity are particularly important for oscillating activity underlying cognition [51]. Cortical interneurons are vulnerable to oxidative stress during development and in mature brain [15,52]. Effects of prenatal stress on both microglia and cortical interneurons in the embryonic brain are ameliorated by maternal administration of NAC or another antioxidant, astaxanthin (AST) [21,40]. AST is a naturally occurring carotenoid that reduces oxidative stress in the brain [53-55]. AST given to adult offspring ameliorates anxiety- and depressive-like behavioral deficits arising from prenatal maternal inflammation [56], but AST has not been used during prenatal stress to determine oxidative stress mechanisms for persistent neurobehavioral outcomes. Studies in which only a single antioxidant agent is used do not provide convergent data to more precisely address oxidative stress as a mechanism, rather than off-target mechanisms of individual agents.

We sought to determine whether oxidative stress during prenatal stress plays a role in its persistent impacts in offspring by examining adult neurobiological and behavioral outcomes with manipulation of oxidative stress during the prenatal period using either NAC or AST, similar to previous work on embryonic brain [21]. These two antioxidants have similar efficacy for reducing oxidative stress but different mechanisms by which this occurs: NAC provides substrate for producing glutathione, a critical component of reducing reactions, but also acts through other non-oxidative stress mechanisms in the brain which may be a confound with use of only NAC to manipulate oxidative stress [57,58]. AST broadly augments endogenous antioxidant processes in multiple ways [59], and other non-oxidative stress processes it may affect are poorly understood. Our study is unique in use of these two different antioxidants to convergently determine the necessity of prenatal oxidative stress. We hypothesized that outcomes due to oxidative stress-related mechanisms would be affected similarly by maternal administration of either, but oxidative stress-related mechanisms were less likely implicated if only one agent has effects. We evaluated cortical microglia and medial frontal cortical and hippocampal GABAergic interneurons with the hypothesis that, because of their sensitivity to oxidative stress, they would be altered in the adult brain by prenatal stress; we also hypothesized that either maternal antioxidant treatment during prenatal stress would prevent these changes as modifiers of prenatal redox mechanisms. Prenatal stress-exposed rodent offspring consistently have altered anxiety-like behavior, social behavior, locomotor activity, and sensorimotor gating [41,42,60-63]. We hypothesized that we would find these same deficits which would also be prevented by either maternal antioxidant. Lastly, we expected that neurobiological and behavioral outcomes would be correlated across all individual animals, given previous find-

ings demonstrating the contribution of microglial morphologies and interneuron populations in these complex behaviors [34,50,51]. The goal of this study of prenatal stress models is to elucidate the critical role of prenatal oxidative stress in neuropsychiatric risk, information that can be used to develop better preventive measures and treatments for those at risk for neuropsychiatric disease.

## METHODS

### *Mice*

GAD67-GFP+/- knock-in mice used to assess GABAergic interneuron populations were bred on a CD1 background, housed, and tested in accordance with our Institutional Animal Care and Use Committee (IACUC) policies. All mice were housed in cages on a 12-hour light/dark cycle with free access to food and water. For timed pregnancies, breeding-naïve GAD67-GFP-/- female mice were bred with GAD67-GFP+/- males. The detection of a vaginal plug established embryonic day 0 (E0). Prenatally-stressed dams were singly housed from E12 onward and non-stressed dams were co-housed.

### *Prenatal Stress and Treatments*

Beginning on E12 and for the duration of their pregnancies, prenatally- and non-stressed dams (PS and NS, respectively) were given intraperitoneal injections of either: 1) N-acetylcysteine (NAC) (once daily 200 mg/kg of 40 mg/mL phosphate buffered saline (PBS) solution with 30% sodium hydroxide (1 μM) to bring pH to 7.4; Sigma A7250) [64-66], 2) astaxanthin (AST; three times daily due to shorter half-life: 30 mg/kg [first daily injection] or 10 mg/kg [second and third daily injections] in a 10mg/mL PBS solution; with 3% dimethyl sulfoxide [DMSO]; Sigma SML0982) [67,68], or 3) PBS alone (once daily 200 μL), 20 minutes prior to restraint stress sessions or at equivalent times in non-stressed dams. Half of pregnant females underwent prenatal stress in a clear, plastic restraint under bright lights for 45 minutes, three times a day, at 3 to 4 hour intervals [63]. All pregnant dams (n=18, 3 per condition) gave birth to between 6-13 pups, with the average litter size of 10 pups. On the day of birth, postnatal day 0 (P0), litters were culled to six to eight with even distribution of male and female pups. On P24, mice were weaned from their mothers and then single-sex group-housed by condition.

### *Behavioral Tests of Adult Offspring*

All behavioral tests were performed on 10-14-week-old male and female mice, representing all offspring from 18 litters. Sample sizes ranged from nine to 12 animals from each condition and sex. During the light cycle, mice were allowed to habituate to the testing room 30 minutes prior to testing. All behavioral tests were conducted on consecutive days with only one test per day in the order listed here in the methods (least to most stressful). Mice remained in their home cage with their cage-mates immediately before and after testing to limit the inherent stress of behavioral testing. All cages were equipped with hoppers that provided food and water for the duration of the testing period. Each testing apparatus was cleaned with 70% ethanol and dried prior to each daily assessment and between the assessment of each mouse.

*Open field (OF)*: In a 1,500 cm2 rectangular, clear plastic arena, offspring mice were tested on the open field test for 30 minutes on two consecutive days. The amount of time spent in the center 50% of the arena was measured. Trials were video-recorded by a suspended, overhead camera and movement data were analyzed using Anymaze software (Stoelting, Wood Dale, Illinois, USA). This task was performed to assess anxiety-like behaviors on Day 1 and locomotor activity on Day 2 in the offspring mice.

*Elevated plus maze (EPM)*: Offspring mice were tested on a plastic Elevated Plus Maze approximately two feet above the ground, consisting of two, 36 cm long closed arms with 20 cm tall walls and two open arms without any walls for 5 minutes on a single day. The amount of time spent in each type of arm, in entries into the closed and open arms, and the center of the maze were video-recorded using Anymaze software. The ratio of time in the open to the closed arms was calculated for each individual. This test was performed to evaluate the anxiety-like behaviors in the offspring mice.

*Social approach*: In a three-chamber sociability and social recognition apparatus, offspring mice were tested for sociability and social recognition on a single day. The clear, plastic apparatus consisted of three, equally-sized 20 x 40 x 22 cm chambers with two 5 x 8 cm doors allowing access to all three chambers. The left- and right-side chambers contained a single 9 cm diameter, 10 cm tall wire cup. The social approach task consisted of three phases. During the first phase, the doors to the other chambers were closed and, for 10 minutes, the experimental mouse was allowed to habituate to the center chamber.

During the second phase or the "sociability" phase, a non-experimental mouse ("mouse") of the same age, sex, and strain was placed under the wire cup of one of the two side chambers. During the third phase or the "social recognition" phase, the same, non-experimental mouse (previously known as "mouse," but denoted the "familiar mouse" during this phase) was placed under the wire cup of the opposite chamber and another mouse ("novel mouse") of the same age, sex, and strain was placed under the wire cup of the other chamber. Each phase lasted for 10 minutes and the movements of the experiment

mouse across all three chambers were video-recorded by Anymaze software. The amount of time spent in close proximity to the original "stranger mouse cup" or "empty cup" and the "novel mouse cup" or "familiar mouse cup" were recorded and statistically analyzed. Social approach task was performed to examine both general sociability and recognition of social novelty. Sociability index was calculated as: time with stranger/total time in close proximity to either stranger mouse or empty cup. Social Recognition index was calculated as: time with novel mouse/ total time in close proximity to either novel or familiar mouse cups.

*Prepulse inhibition*: Mice were place into a Plexiglas cylinder and secured onto the platform in a lighted, soundproof chamber, the SR-LAB startle apparatus. A piezoelectric accelerometer measured and recorded the startle reflex response. A loudspeaker mounted within the chambers generated the startling acoustic stimuli as well as the ambient, 70-dB white background noise. The sessions began with a 5-minute habituation period and then proceeds with 90 different trials broken up over three blocks over the course of a single day. The first block consisted of five 20msec 120 dB pulse alone (no prepulse) trials. The second block consisted of 10 randomized trials of pulse alone, 5 dB, 10 dB, and 15 dB prepulses before the 120dB pulse. The third block consisted of a final block of five consecutive pulse-alone trials. Deficits in sensorimotor gating were assessed by calculating the prepulse inhibition percentage (PPI%, calculated as: 100 x [startle reflex from acoustic pulse alone – startle reflex from prepulse-elicited stimulus (5, 10, or 15 dB)] / startle reflex from acoustic pulse alone).

### *Immunohistochemistry*

Brain tissue from the GAD67GFP+/- offspring adult offspring mice from behavioral testing were used for immunohistochemistry. Sample sizes ranged from two to five animals from each condition and sex across at least two litters and typically representing all three litters per condition. Four weeks after the final day of behavioral testing, to avoid effects of testing stress, brains were collected for examination. Adult offspring mice were perfused first with cold PBS and then with 4% paraformaldehyde. Brains were collected from male and female mice and placed directly into 4% paraformaldehyde. After overnight paraformaldehyde incubation, brains were rinsed in PBS and transferred to 20% sucrose for at least 20 hours. Brains were embedded in optimal cutting temperature compound, coronally cryosectioned at 50 µm, and then incubated in 10% goat serum/PBS++ blocking solution (with 0.025% Triton X-100, 0.0125% Tween 20) at room temperature for at least 1 hour. Immunohistochemistry was performed with 5% goat serum/PBS++ and primary antibodies, anti-Iba1 (1:500, WAKO; #019-19741), anti-PV (1:1000, Sigma Aldrich; SAB4200545) and anti-GFP (1:1000, Abcam, AB13970), and allowed to incubate overnight. Sections were stained with the Alexa dye-conjugated secondary antibody (1:500–1000; Molecular Probes) in 5% goat serum/PBS++ incubation for 2 hours. Tissue section slides were then cover slipped using DAPI mounting medium (Vector Laboratories, #H-1200).

### *Cell Counting*

Stereological estimates of total cortical densities of Iba1+ microglia and hippocampal cornus ammonis (hippocampus) and medial frontal cortex (mFC) densities of GAD67GFP+ cells and PV+ cells were calculated using an optical fractionator approach and unbiased counting rules with 3-dimensional counting frames: 150 × 100 × 10 µm counting frames, on a 1000 x 600 µm grid for total neocortex, 450 × 450 µm grid for mFC and 600 x 600 µm grid for hippocampus, using a 40× objective lens (Stereoinvestigator; MBF Biosciences, Williston, VT, USA). Stereological counting to determine cell density, displayed as means and standard errors of the mean, was performed in 3 to 8 serial coronal sections (every 20$^{th}$ section of the neocortex; every 10th section of the mFC and hippocampus) as previously described [41,44,63]. Microglia morphologies were defined as: amoeboid (those with zero to one process), lowly ramified (those with two or three processes) which may reflect an activated state, moderately ramified (those with four processes or those with multiple thin, spindly processes and a small soma), and highly ramified (those with five or more processes and a large soma, also referred to as bushy microglia) which may reflect a primed state. Iba1+, GAD67GFP+, and PV+ cells in coronal tissue sections was counted using fluorescent microscopy with a Zeiss Axiolmager M2 microscope.

### *Data Analysis*

*A priori* two-tailed student's t-tests were used in each assessment to first detect any baseline differences between the control non-stressed and control prenatal stress groups (non-stressed PBS vs. prenatally-stressed PBS). For prepulse inhibition data, *a priori* ANOVA across intensities was used. Two-way ANOVAs were also used to evaluate the effects of prenatal stress and each antioxidant manipulation independently for each assessment, looking for main effects and interactions that would indicate the ability of each antioxidant treatment manipulation to significantly modify effects of prenatal stress. When appropriate, *post-hoc* Bonferroni tests were performed to determine specific group differences, correcting for multiple comparisons. Because of the relatively small sample sizes of some of the groups, trending significant values of

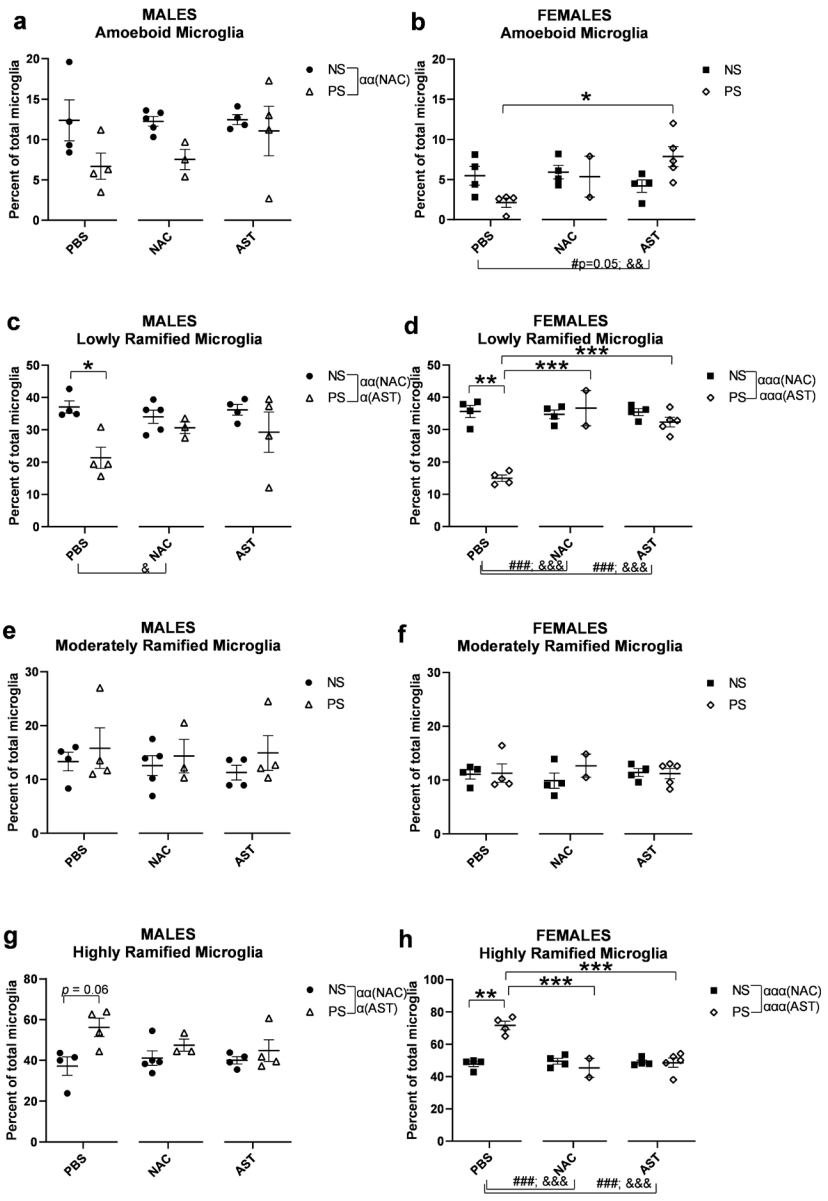


**Figure 1. Prenatal stress decreased lowly ramified and increased highly ramified cortical microglia, prevented by antioxidants.** Prenatal stress (PS) compared to non-stressed (NS) condition reduced amoeboid microglia in male (**a**) and female (**b**) mice (ANOVA main effects across PBS and NAC male groups αα $p<0.01$ and across PBS and AST female groups α $p<0.05$). AST trend increased female amoeboid microglia (ANOVA main effect of AST # $p=0.05$). Prenatal stress and AST interacted to in effects on female amoeboid microglia (&& $p<0.01$ two-way ANOVA), demonstrating AST prevention from of stress effects (*post-hoc* test * $p<0.05$). Prenatal stress also reduced lowly ramified microglia in male (**c**) and female (**d**) mice (*a priori* t-tests * $p<0.05$ in males, ** $p<0.01$ in females; ANOVA main effects across PBS and NAC or AST groups: α $p< 0.05$, αα $p<0.01$, ααα $p<0.001$) with prevention by NAC for males (ANOVA interaction & $p<0.05$) and AST and NAC for females (ANOVA interactions &&& $p<0.001$, *post-hoc* tests *** $p<0.05$). NAC and AST both increased lowly ramified microglia (ANOVA main effects for NAT and AST ### $p<0.001$). No effects on moderately ramified microglia were found in males (**e**) or females (**f**). Highly ramified microglia were increased by prenatal stress in males (**g**) and females (**h**) (*a priori* t-tests males $p=0.06$; females: ** $p<0.01$: ANOVA main effects across PBS and NAC or AST groups: α $p<0.05$, αα $p<0.01$, ααα $p<0.001$). NAC and AST prevented this effect in females (ANOVA interaction of prenatal stress NAC or AST ααα $p<0.001$; *post-hoc* tests *** $p<0.001$). NAC and AST both reduced highly ramified microglia (ANOVA main effect of NAC or AST, ### $p<0.001$). Means and Standard errors of the mean are displayed.

$p \leq 0.07$ were reported to avoid type II error.

## RESULTS

### Cortical Microglia Populations

A shift in cortical microglia morphology with prenatal stress in male offspring has been previously shown [38,41]. Here, prenatal stress led to this same effect in both male and female offspring (Figure 1a-h). In males, percent of amoeboid and lowly ramified microglial morphologies were reduced by prenatal stress and highly ramified microglia morphology was increased in the neocortex (Figure 1a, c, g, *a priori* t-tests: lowly ramified $p<0.05$; highly ramified $p=0.06$; ANOVA stress main effects: amoeboid across PBS and NAC $F[1,12]=10.00$, $p=0.008$, lowly ramified across PBS and NAC $F[1,12]=15.18$, $p=0.002$ and PBS and AST $F[1,12]=9.141$, $p=0.0106$; highly ramified across PBS and NAC $F[1,12]=9.383$, $p=0.0098$ and PBS and AST $F[1,12]=7.673$, $p=0.017$). The reduced percent of lowly ramified microglia with prenatal stress in males was prevented by NAC (Figure 1c, ANOVA interaction: $F[1,12]=6.284$, $p=0.0276$).

In females, the neocortical percentages of lowly ramified microglia was also decreased and highly ramified increased by prenatal stress (Figure 1b, d, *a priori* t-test: lowly ramified $p=0.0019$, high ramified $p=0.0074$; ANOVA stress main effects: lowly ramified across PBS and NAC $F[1,10]=20.60$, $p=0.0011$ and across PBS and AST $F[1,13]=67.91$, $p<0.0001$, high ramified across PBS and NAC $F[1,10]=13.72$, $p=0.0004$ and across PBS and AST $F[1,13]=25.65$, $p=0.0002$). All prenatal stress effects on microglial morphology in female offspring were prevented by NAC or AST (Figure 1b, d, f, h, ANOVA stress interactions with NAC lowly $F[1,10]=29.92$, $p=0.0003$, *post-hoc* PBS PS vs NAC PS $p=0.003$, highly $F[1,10]=27.47$, $p=0.0003$, *post-hoc* PBS PS vs NAC PS $p=0.005$; AST amoeboid $F[1,13]=11.43$, $p=0.0049$, *post-hoc* PBS PS vs AST PS $p=0.016$, lowly $F[1,13]=36.89$, $p<0.0001$, *post-hoc* PBS PS vs AST PS $p$,0.0001, highly $F[1,13]=27.32$, $p=0.0002$, *post-hoc* PBS PS vs AST PS $p<0.0001$). Total cortical microglia density was not affected in males or females by stress, NAC, or AST (data not shown).

### Medial Frontal Cortical GABAergic Populations

A larger proportion of parvalbumin (PV) subtype of GABAergic interneurons has been found in medial frontal cortex (mFC) of prenatally-stressed male offspring [63]. Here, prenatal stress did not show this effect in male offspring mFC *a priori*, but when the effect of prenatal stress was assessed across both PBS and AST groups, this same effect was found (Figure 2a, ANOVA main effect: $F[1,13]=9.13$, $p=0.009$). Furthermore, there was an interaction between the effects of prenatal stress and NAC administration on PV+/GAD67+ cell ratio in mFC ($F[1,13]=.84$, $p=0.01$); NAC prevented the increase of the PV subtype. Prenatal stress in PBS exposed mothers resulted in an increased male offspring ratio of PV+/GAD67+, but in NAC exposed mothers, decreased the PV+/GAD67+ cell ratio in the male offspring mFC (Figure 2a). Antioxidants overall decreased the ratio of PV+/GAD67+ cells in the mFC compared to PBS (NAC: $F[1,13]=18.89$, $p=0.002$ and AST: $F[1,13]=4.71$, $p=0.04$), suggesting prenatal redox down-regulation reduced maturation of cortical interneurons into this subtype in male offspring. This may have been affected by a trend increased density of total GAD67+ cells with AST ($F[1,14]=3.92$, $p=0.07$, Figure 2c) and a trend decreased density of PV+ cells with NAC ($F[1,13]=4.62$, $p=0.05$, Figure 2e).

In female offspring, prenatal stress induced the opposite effect, decreasing the PV+/GAD67+ cell ratio in the mFC (*a priori* t-test: $p=0.005$, ANOVA main effect across PBS and NAC: $F[1,10]=8.46$, $p=0.01$; Figure 2d). AST administration interacted with prenatal stress (ANOVA: $F[1,12]=6.40$, $p=0.03$), suggesting prevention of the prenatal stress effect. These effects in females were mainly due to altered PV+ cell densities in the mFC (Figure 2f). *A priori* t-test showed that prenatal stress reduced PV+ cells in the mFC ($p=0.009$, Figure 2f). Interactions of NAC and AST with prenatal stress effects on PV+ cell density was observed (NAC: $F[1,10]=7.25$, $p=0.02$, and AST: $F[1,13]=7.30$, $p=0.02$) suggesting that the decrease with stress did not occur when prenatal antioxidants buffered oxidative stress. An interaction of NAC treatment and prenatal stress was also found for GAD67+ total density ($F[1,10]=6.28$), $p=0.03$, Figure 2d).

### Hippocampal GABAergic Populations

No difference was found in male offspring hippocampal PV+/GAD67+ cell proportion due to prenatal stress by *a priori* assessment or analysis across groups (Figure 3a). NAC groups, but not AST groups, decreased the PV+-to-GAD67+ cell ratio in the male hippocampus ($F[1,10]=6.28$, $p=0.03$) similar to its effects in the mFC. Prenatal stress decreased GAD67+ cell density (*a priori* t-test: $p=0.07$; ANOVA main effect across PBS and NAC: $F[1,14]=11.43$, $p=0.008$, Figure 3c). AST and prenatal stress interacted ($F[1,15]=5.01$, $p=0.04$) to show that the decrease of hippocampal GAD67+ cell density with prenatal stress trend returned to the non-stressed level (*post-hoc*, trend sig., $p=0.07$). No differences in PV+ cell densities were detected in the hippocampal CA between any of the groups of male offspring (Figure 3e).

In female offspring, prenatal stress decreased the PV+/GAD67+ cell ratio in the hippocampus as in the mPFC (*a priori* t-test: $p=0.04$; ANOVA main effect across PBS and AST: $F[1,12]=23.08$, $p=0.001$, Figure 3b). NAC

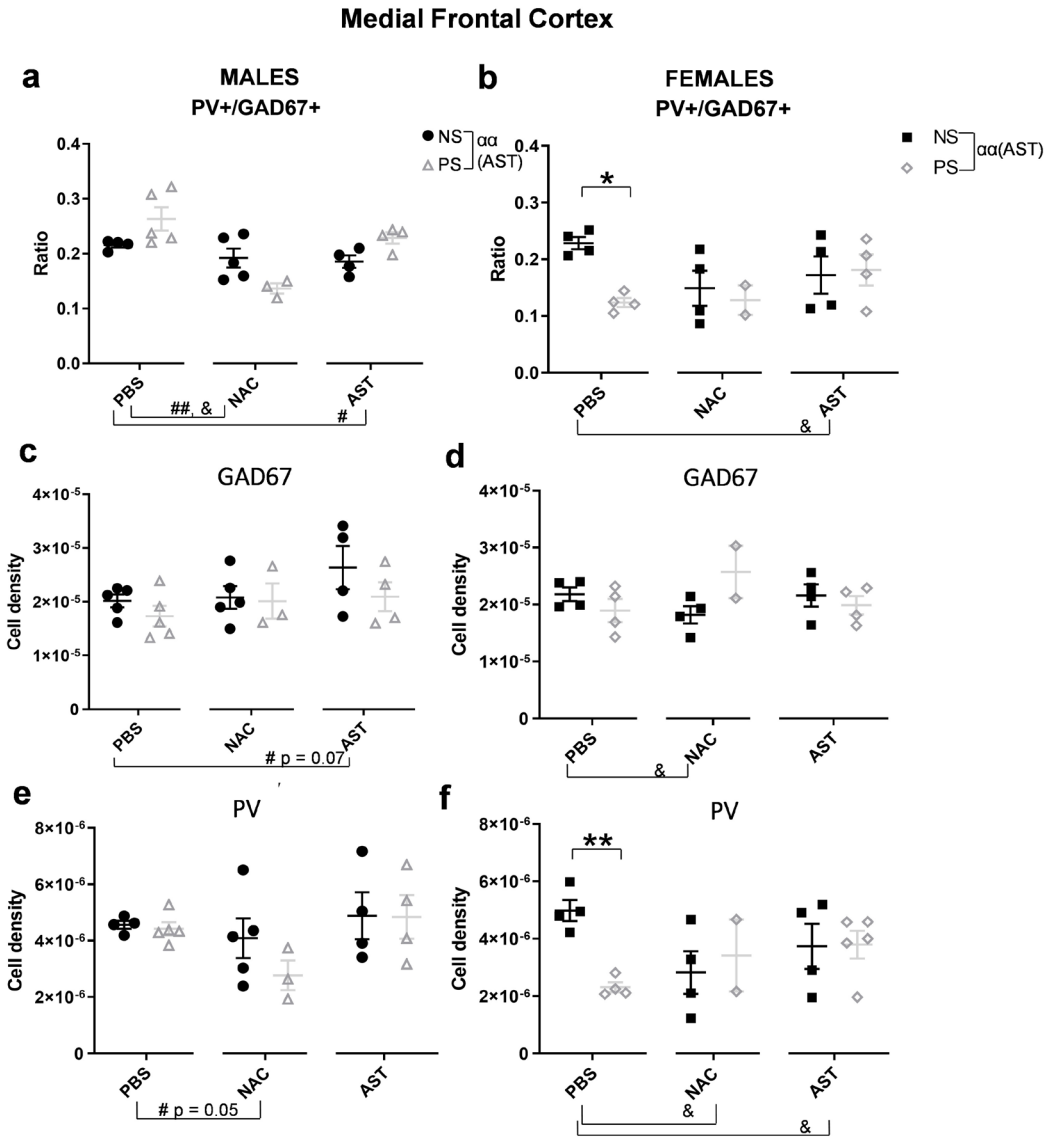


**Figure 2. Prenatal stress increased the PV+/GAD67+ cell ratio in mFC in male offspring and decreased this ratio in females. NAC and AST prevented these impacts in males and females respectively.** (**a**) Prenatally-stressed (PS) compared to non-stressed (NS) male mice had a higher ratio of PV+/GAD67+ cells in the mFC (αα $p<0.01$ main stress effect by two-way ANOVA across PBS and AST groups). A prenatal stress by NAC interaction was found in PV+/GAD67+ cells (& $p<0.05$ by two-way ANOVA). A *post-hoc* test revealed a significant decrease in PV+/GAD67+ proportion between the PS PBS and the PS NAC mice (* $p<0.05$). NAC and AST decreased the ratio of PV+/GAD67+ cells in the mFC compared to PBS (## $p<0.01$ and # $p<0.05$ by two-way ANOVA, respectively). (**b**) *A priori* t-test found that PS PBS female mice had a lower ratio of PV+/GAD67+ cells in the mFC ($$ $p<0.01$ by t-test). Prenatal stress led to lower ratios of PV+/GAD67+ cells in female mice as revealed by a main effect of stress (α $p<0.05$ by two-way ANOVA across PBS and NAC groups). An interaction between the effects of prenatal stress and maternal AST treatments on the ratio of PV+/GAD67+ cells (& $p<0.05$) suggests that AST prevented this impact of prenatal stress. (**c**) AST males had trend higher densities of GAD67+ cells in the mFC than PBS males (trend sig., $p=0.07$ by two-way ANOVA). (**d**) An interaction of NAC treatment and prenatal stress was found for female mPFC GAD67+ density (& $p<0.05$ by two-way ANOVA). (**e**) NAC-treated offspring had lower densities of PV+ cells compared to PBS offspring (trend sig., $p=0.05$ by two-way ANOVA). (**f**) *A priori* t-test found that PS PBS female mice had a lower density of PV+ cells in the mFC (** $p<0.01$ by t-test). Interactions of NAC and AST with prenatal stress effects on PV+ cells were observed (& $p<0.05$, respectively), suggesting that these antioxidants prevented the effect of prenatal stress. Means and Standard errors of the mean are displayed.

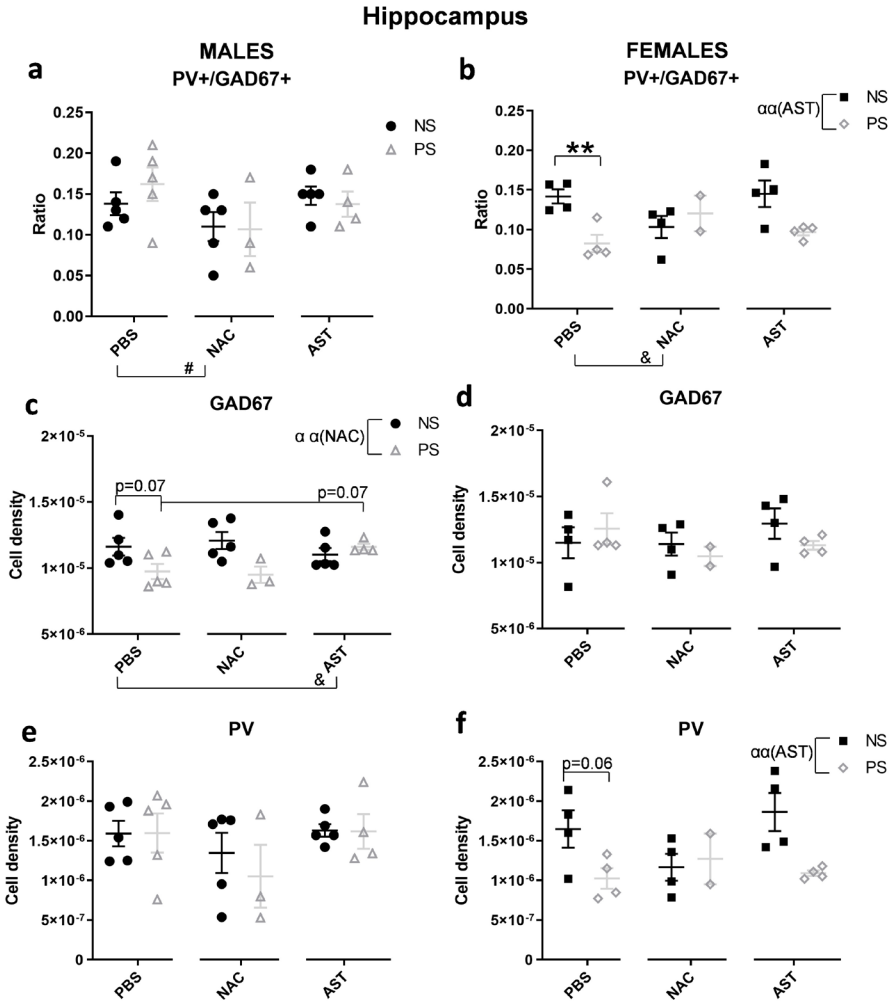


**Figure 3. Prenatal stress also decreased PV+/GAD67+ ratio in female hippocampus, prevented by NAC.** (**a**) No baseline differences were detected in prenatal stress (PS) PBS and non-stressed (NS) PBS male offspring PV+/GAD67+ cell proportion and no main effect of prenatal stress was found. Maternal treatments of NAC decreased the PV+-to-GAD67+ cell ratios in the male hippocampal CA (# $p$<0.05 by two-way ANOVA). (**b**) *A priori* t-test found that PS PBS females had a lower proportion of PV+/GAD67+ cells in the hippocampal CA compared to NS PBS female mice (** $p$<0.01 by t-test). Prenatal stress led to lower PV+/GAD67+ cell ratios in female mice as revealed by a main effect of stress (αα $p$<0.01 by two-way ANOVA). An interaction of prenatal stress effects and maternal treatment of NAC (& $p$<0.05 by two-way ANOVA) revealed that effects of prenatal stress on the PV+/GAD67+ ratio was prevented by NAC. AST did not prevent the effects of prenatal stress and PS AST mice had significantly lower PV+/GAD67+ ratios compared to NS AST mice (& $p$<0.05). (**c**) *A priori* t-test showed that PS PBS male mice had a trend lower GAD67+ cell density than NS PBS mice (trend sig., $p$=0.07). Prenatal stress led to lower GAD67+ cell densities in male mice as revealed by a main effect of stress (αα $p$<0.01 by two-way ANOVA across PBD and NAC groups). An interaction of prenatal stress effects and maternal treatment of AST (& $p$<0.05 by two-way ANOVA) revealed that AST prevented the effect of prenatal stress on GAD67+ density in hippocampus. *Post-hoc* tests revealed a trend increase in GAD67+ cell density in PS AST hippocampus compared to PS PBS hippocampus (trend sig., $p$=0.07). (**d**) No baseline differences in the densities of GAD67+ cells in control (PBS) female mice and no main effects of stress were found. (**e**) No differences in PV+ cell densities were detected in the hippocampus between any of the groups of male offspring mice. (**f**) *A priori* t-test found that PS PBS female mice had a trend lower proportion of PV+ cells in the hippocampal CA (trend sig., $p$=0.06). AST-treated offspring mice had PV+ cell densities similar to the NS PBS and PS PBS mice, revealing a main effect of prenatal stress (αα $p$<0.01 by two-way ANOVA). AST did not rescue the effects of prenatal stress and PS AST mice had significantly lower densities of PV+ cells compared to NS AST mice as revealed by a *post-hoc* test (& $p$<0.05). Means and Standard errors of the mean are displayed.

interacted with prenatal stress effects (F[1,10]=8.13, $p$=0.04) suggesting prevention of this effect by NAC. While there were no differences in GAD67+ cell density, prenatal-stress reduced PV+ cell density in the hippocampus (*a priori* t-test: trend sig., $p$=0.06; ANOVA main effect across PBS and AST: F[1,12]=14.77, $p$=0.008; Figure 3f), suggesting that prenatal stress impaired parvalbumin cell maturation in females.

### *Anxiety-like Behavior*

We found generally that prenatal stress increased anxiety-like behavior in offspring. PS PBS male mice compared to NS PBS controls had decreased ratio of open:closed arm time of the EPM, suggesting an anxiety-like phenotype (*a priori* t-test $p$=0.03, Figure 4a). Prenatal stress decreased ratio of open:closed arm time across both PBS and NAC groups, demonstrating that NAC manipulation did not return anxiety-like behavior to normal levels (ANOVA time ratio across PBS and NAC: F[1,44]=4.45, $p$=0.04; Figure 4a). Similar outcomes were shown for male offspring in closed arm time (*a priori* t-test $p$=0.048; ANOVA main effect across PBS and NAC: F[1,44]=8.18, $p$=0.006. Data not shown). The ratio of open to closed arm entries also demonstrated an effect of stress (Figure 4c, ANOVA main effect of stress across PBS and NAC: F[1,44]=5.55, $p$=0.02). This main effect of stress was not found across AST groups, but no interactions for AST and stress were present to address moderation of effect either. No differences were found in open arm time or entries for male offspring (data not shown).

For females, prenatal stress produced an anxiety-like phenotype in the ratio of open to closed arm time (Figure 4b, ANPVA main effect of stress across PBS and AST: F[1,41]=4.92, $p$=0.03). There were no differences in open or closed arm time themselves (data not shown). Ratio of open to closed arm entries also indicated anxiety-like behavior by both *a priori* assessment and comparisons across multiple groups (Figure 4d, t-test $p$=0.04, ANOVA main effects of stress across PBS and NAC: F[1,41]= 6.36, $p$=0.02; and across PBS and AST: F[1,39]=4.87, $p$=0.03).

Another anxiety-like behavior measure was made examining time in the center of the open field in the first 5 minutes on the first day (Figure 4e, f). No difference was detected between prenatally-stressed and non-stressed PBS male or female mice based on the *a priori* t-test, and no main effects of prenatal stress were found (Figure 4e, f). Neither NAC nor AST had significant effects on this anxiety-like measure in females (Figure 4f), but both maternal antioxidants, NAC and AST, reduced anxiety-like behavior in males overall (increased center time, ANOVA for NAC: F[1,41]=9.44, $p$=0.004; AST: F[1,40]=5.57, $p$=0.02, *post-hoc* PBS PS vs AST PS $p$=0.04; Figure 4e), suggesting an impact of prenatal redox changes in affecting later anxiety-like behavior.

### *Locomotor Activity*

Movement in the open field on the second day was used to assess locomotor activity in offspring. With *a priori* t-tests, no baseline differences were detected in prenatally-stressed and non-stressed control (PBS) male or female offspring, and no overall effect of prenatal stress across groups was found (Figure 4g, h). However, in males, AST administration and prenatal stress had a trend interaction (trend sig., F[1,41]=3.61, $p$=0.06, Figure 4g), suggesting that prenatal AST specifically increased male locomotor activity when combined with prenatal stress exposure.

In females, NAC administration and prenatal stress interacted (F[1,41]=8.57, $p$=0.006, Figure 4h), showing that prenatal NAC increased locomotor activity in non-stressed female mice (*post-hoc*, $p$=0.003).

No differences were found across groups in adult body weight for male or female offspring (data not shown).

### *Sociability*

Prenatal stress did not reduce sociability in male offspring by sociability index, total time spent with a stranger mouse by *a priori* tests, or comparisons across group (Figure 5a, c). Prenatally-stressed male offspring in both PBS and NAC groups spent less time with the empty cup (ANOVA main effect of stress across PBS and NAC: F[1,43]=5.72, $p$=0.02; Figure 5a).

Female prenatally-stressed offspring did have lower sociability by *a priori* comparison of the sociability index similar to previous findings in males (Figure 5f) [63]. Across PBS and AST groups, time spent with a stranger mouse trended lower with prenatal stress (ANOVA main effect: F[1,42]=3.96, $p$=0.065; Figure 5e). NAC and AST also impacted time females spent with the empty cup, shown by an interaction of NAC or AST with prenatal stress (ANOVA: NAC F[1,41]=6.76, $p$=0.01 and AST F[1,42]=5.84, $p$=0.02, Figure 5e). Empty cup time is an important factor in calculating the sociability index, but antioxidants did not statistically interact with prenatal stress on the index itself (Figure 5f) so the reduced female sociability deficit was not sufficiently affected by antioxidants to show prevention.

### *Social Recognition*

No differences in social recognition were found due to prenatal stress in male or female offspring by *a priori* comparisons or main effect across groups (Figure 5g-l). This was evident in the lack of differences found across groups in recognition indices or time spent with

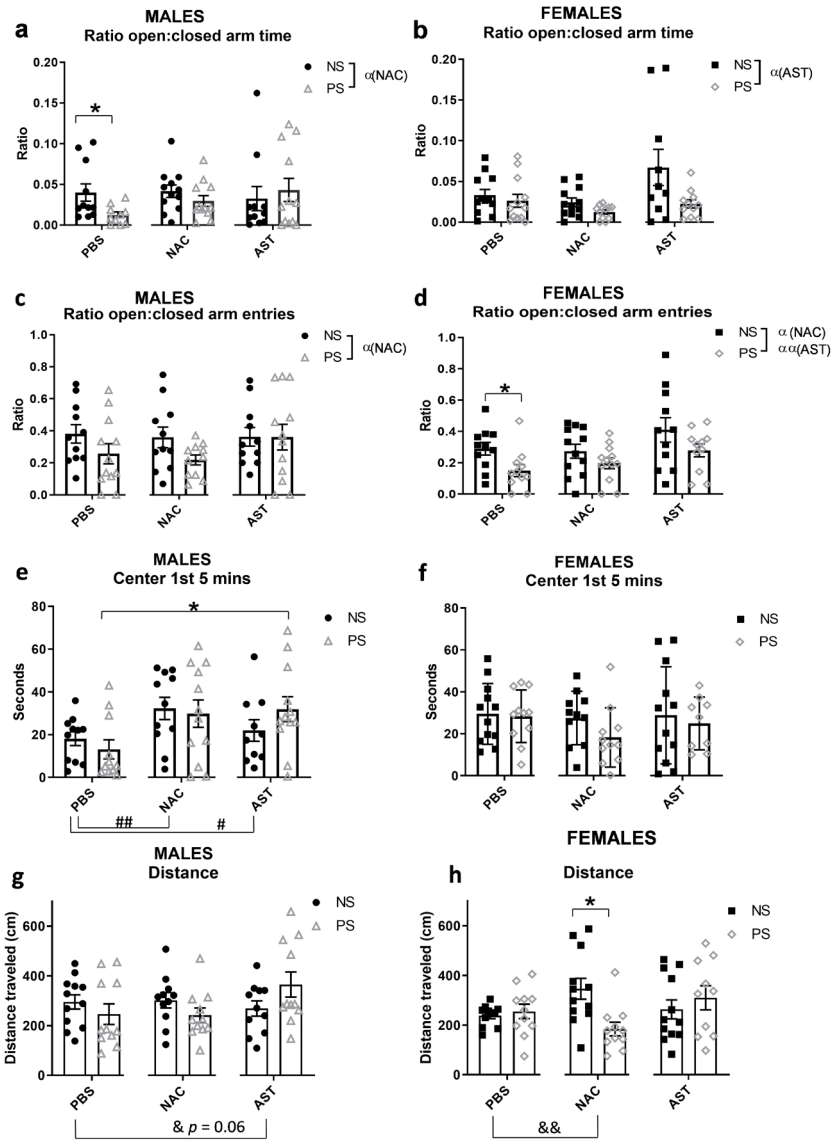


**Figure 4. Prenatal stress increased anxiety-like behavior on the EPM.** (**a**) *A priori* difference of prenatal stress (PS) versus non-stressed (NS) condition in the PBS groups (* t-test $p<0.05$) and main effect of stress in the ratio of time spent in the open arm to the closed arm of the EPM (α $p<0.05$ by two-way ANOVA across PBS and NAC groups). (**b**) Prenatally-stressed females had lower open to closed arm time ratio, an anxious-like phenotype (α $p<0.05$ by two-way ANOVA across PBS and AST groups). (**c**) A main effect of stress was found in the ratio of open:closed arm entries in male mice (α $p<0.05$ by two-way ANOVA across PBS and NAC groups). (**d**) *A priori* t-test found that PS PBS female mice had a lower open:closed arm entry ratio, also an anxious-like phenotype (* $p<0.05$). Prenatally-stressed mice had fewer open:closed arm entries compared to non-stressed female mice (main effects of PBS groups with NAC groups: αα $p<0.01$ and AST groups: α $p<0.05$ by two-way ANOVAs). (**e**) No prenatal stress baseline differences or main effects of stress in male offspring were found**,** but NAC and AST increased time in the center of the open field (## $p<0.01$ and # $p<0.05$, respectively by two-way ANOVA and *post-hoc* * $p<0.05$ compared to PS PBS). (**f**) No differences due to prenatal stress or antioxidant administration in female offspring were found. (**g**) A trend interaction of AST and prenatal stress effects was found (trend sig., & $p=0.06$ by two-way ANOVA), suggesting AST may affect locomotor activity differently depending on whether the mouse experienced prenatal stress. (**h**) No baseline differences were detected in PS PBS and NS PBS female mice and no main effects of prenatal stress were found. An interaction of NAC with prenatal stress effects was revealed (&& $p<0.01$ by two-way ANOVA). NS NAC female mice exhibited increased locomotor activity compared to PS NAC (*post-hoc* * $p<0.05$). Means and Standard errors of the mean are displayed.

novel stranger mice. Of note, female offspring showed a trend interaction of NAC with stress on time with the novel stranger (Figure 5j, ANOVA: F[1,41]=4.0, $p$=0.06) and those exposed to AST spent less time with the familiar stranger (Figure 5k, l, familiar cup ANOVA: F[1,42]=5.81, $p$=0.02), but antioxidants did not affect male social recognition in any way.

### *Sensorimotor Gating*

No baseline differences in startle response in males or females were found across groups (Figure 6a, b). Male PBS offspring showed reduced pre-pulse inhibition (PPI) across all intensities by prenatal stress with *a priori* ANOVA (F[1,62]=4.77, $p$=0.03, Figure 6c). AST, but not NAC, interacted with prenatal stress effects on PPI in males at all intensities showing prevention of stress effects (ANOVAs: 5 dB: F[1,42]=4.64, $p$=0.04; 10 dB: F[1, 40]=8.20, $p$=0.007; 15 dB: F[1,41]=8.80, $p$=0.005; *post-hoc* PBS PS vs AST PS 10 dB: $p$=0.07). AST also showed an effect in reducing sensorimotor gating in non-stressed males (15 dB: *post-hoc* PBS NS group vs AST NS group $p$=0.04).

In female offspring, there was no effect of prenatal stress on PPI across or at any specific intensity. When all groups were analyzed, AST and NAC both had an independent effect of increasing sensorimotor gating across non-stressed and prenatally-stressed females at some intensities (main effects: NAC 10 dB: F[1,42]=4.27, $p$=0.04; NAC 15 dB: F[1,42]=4.23, $p$= 0.04; AST 5 dB: F[1,41]=13.57, $p$=0.007; Figure 6d). A notable specific finding in females was significantly higher PPI in prenatally-stressed offspring with NAC and AST at the lowest intensity (5dB NAC: ANOVA interaction F[1,42]=10.52, $p$=0.002 and *post-hoc*, PBS PS vs NAC PS: $p$=0.04 and NAC NS vs PBS PS $p$=0.02; PBS PS vs AST PS: $p$=0.01).

### *Correlations of Behavior and GABAergic Populations*

All results were synthesized in Table 1. Some correlations were found between mFC and hippocampal GABAergic populations and behavior across individual animals. In male mice, lower hippocampal GAD67+ cell density trend correlated with decreased open field center time ($R$=0.36, $p$=0.07) as found previously [63]. For females, higher GAD67+ cell density in the mFC correlated with reduced open field center time ($R$=-0.67, $p$=0.002) and trend correlated with increased closed arm time of the EPM ($R$=0.41, $p$=0.06). Higher female hippocampus GAD67+ cell density correlated with greater social recognition ($R$=0.46, $p$=0.03). Notably, GAD67+ cell density, social recognition, EPM closed arm time and open field center time were not altered by stress or treatment in females, but GAD67+ cell density was reduced by stress in males in hippocampus. No correlations were found of behavioral outcomes with cortical microglia morphology.

## DISCUSSION

In this study, we assessed the hypothesis that effects on offspring brain and behavior from prenatal stress are due to oxidative stress that occurs during pregnancy. Specifically, we predicted that prenatal stress effects in male and female mouse offspring would be prevented by the administration to pregnant dams of either N-acetylcysteine or astaxanthin, two antioxidants with different mechanisms. We confirmed this hypothesis for offspring cortical microglia morphology and interneuron population effects. Female offspring had a clear increase in highly ramified microglia morphologies after prenatal stress and both antioxidants prevented these effects (Figure 1). Similarly, for female medial frontal cortical interneuron populations, prenatal stress decreased the density of the parvalbumin interneuron subtype and this effect was prevented by either antioxidant (Figure 2). Male neurobiological outcomes after prenatal stress and their prevention by maternal antioxidant were also found but were less clear and less consistent (Figures 1-3). For behavioral outcomes with relevance to neuropsychiatric disorders, the prenatal stress effect in male offspring in decreasing sensorimotor gating, as measured through prepulse inhibition, was the only behavioral impact prevented through maternal antioxidant administration, specifically with N-acetylcysteine (Figure 6). Other effects on anxiety-like and social behaviors were not prevented by either antioxidant (Figures 4,5).

The dissociation we found of prenatal-stress induced neurobiological (antioxidant prevented) and behavioral (little effect of antioxidants) outcomes in terms of their involvement of prenatal oxidative stress is interesting. First, it is important to acknowledge that regardless of offspring sex, we found that antioxidants had clear impacts on offspring suggesting that both types and dosing used were biologically relevant. We used the same dosing as in previous studies demonstrating a clear impact on these same neurobiological domains in embryonic brain after prenatal stress [21,40]. Interestingly, for outcomes affected by prenatal stress, we found a trend correlation between decreased hippocampal GAD67+ cells and increased anxiety-like behavior in male offspring, suggesting a connection that has been made previously [47,63] and deserves continued investigation. However, results do not generally support the hypothesis that microglia morphology and interneuron populations underlie changes in anxiety-like, social, and sensorimotor gating behavior. Most behavioral outcomes were not correlated with neurobiological measures, and effects of prenatal stress on anxiety-like and social behavior persisted de-

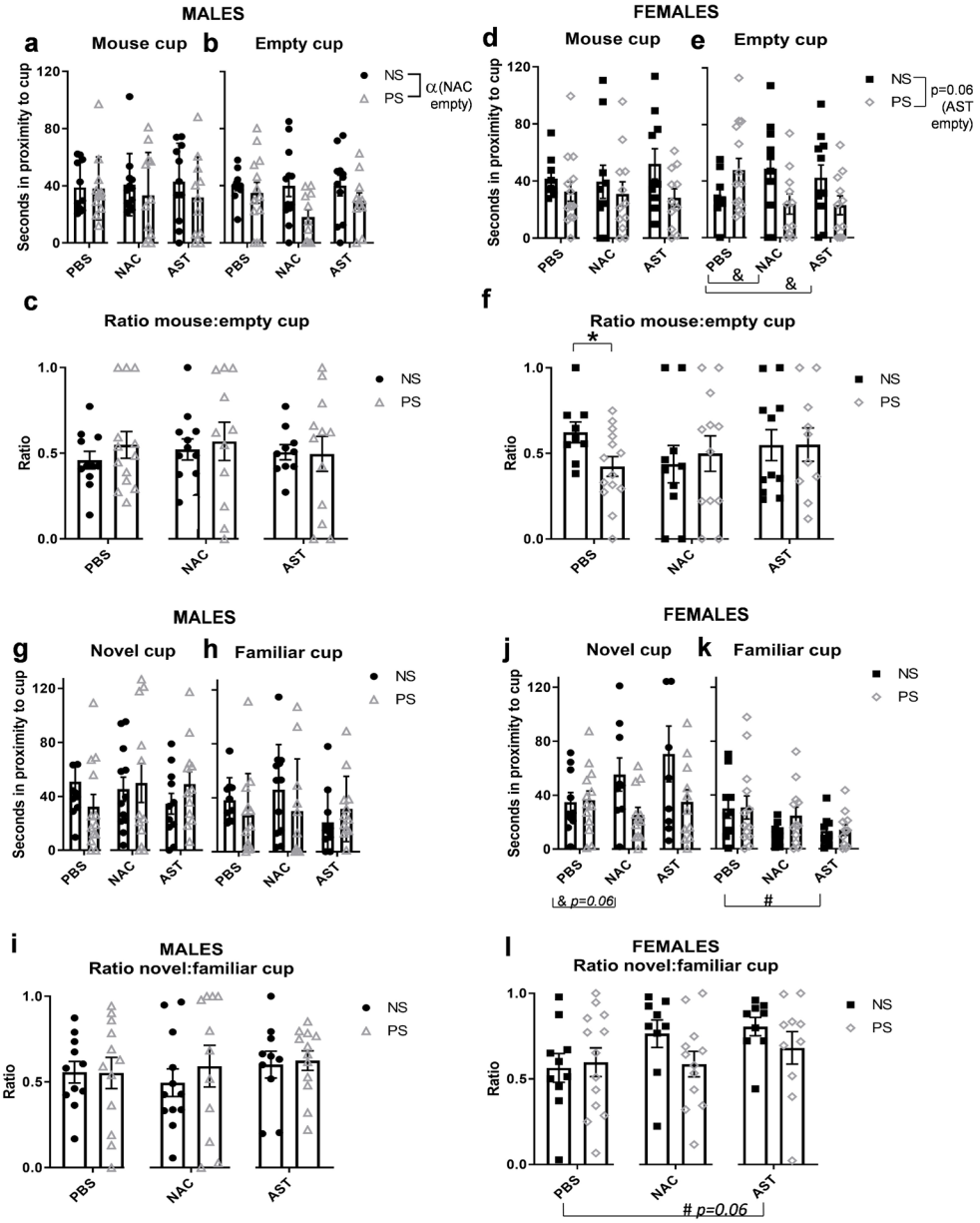


**Figure 5. Sociability but not social recognition was reduced by prenatal stress in female mice.** (**a**) No male differences in time spent in proximity to the mouse cup. (**b**) Prenatally-stressed (PS) compared to non-stressed (NS) male mice spent less time with the empty cup compared to non-stress mice (α $p<0.05$ by two-way ANOVA comparing across PBS and NAC groups). (**c**) No male differences in the ratio of time spent with the mouse cup to time spent with the empty cup. (**d**) There were no differences in the time with the mouse cup across female offspring groups. (**e**) Interactions of prenatal stress with NAC and AST show that impacts of prenatal stress on empty cup time are reversed by NAC and AST (& $p<0.05$ by two-way ANOVAs). (**f**) *A priori* difference was found in the ratio of time spent with the mouse cup to the empty cup by prenatal stress (* $p<0.05$) but no other differences across groups. (**g**) No prenatal stress baseline differences or main effects of stress in male offspring behavior were found for time spent with novel mouse, (**h**) time spent with the familiar mouse, or (**in**) time with novel:familiar mouse. (**j**) While no prenatal stress baseline differences or main effects of stress in female offspring were observed in time spent with the novel or (**k**) familiar mouse, NAC trend interacted with stress for effects on time with novel mouse (& $p=0.06$ trend interaction by ANOVA) and maternally-treated AST female mice spent less time with the familiar mice (# $p<0.05$ by two-way ANOVA). (**l**) ratio of time spent with novel:familiar in female offspring mice trended high with AST. Means and Standard errors of the mean are displayed.

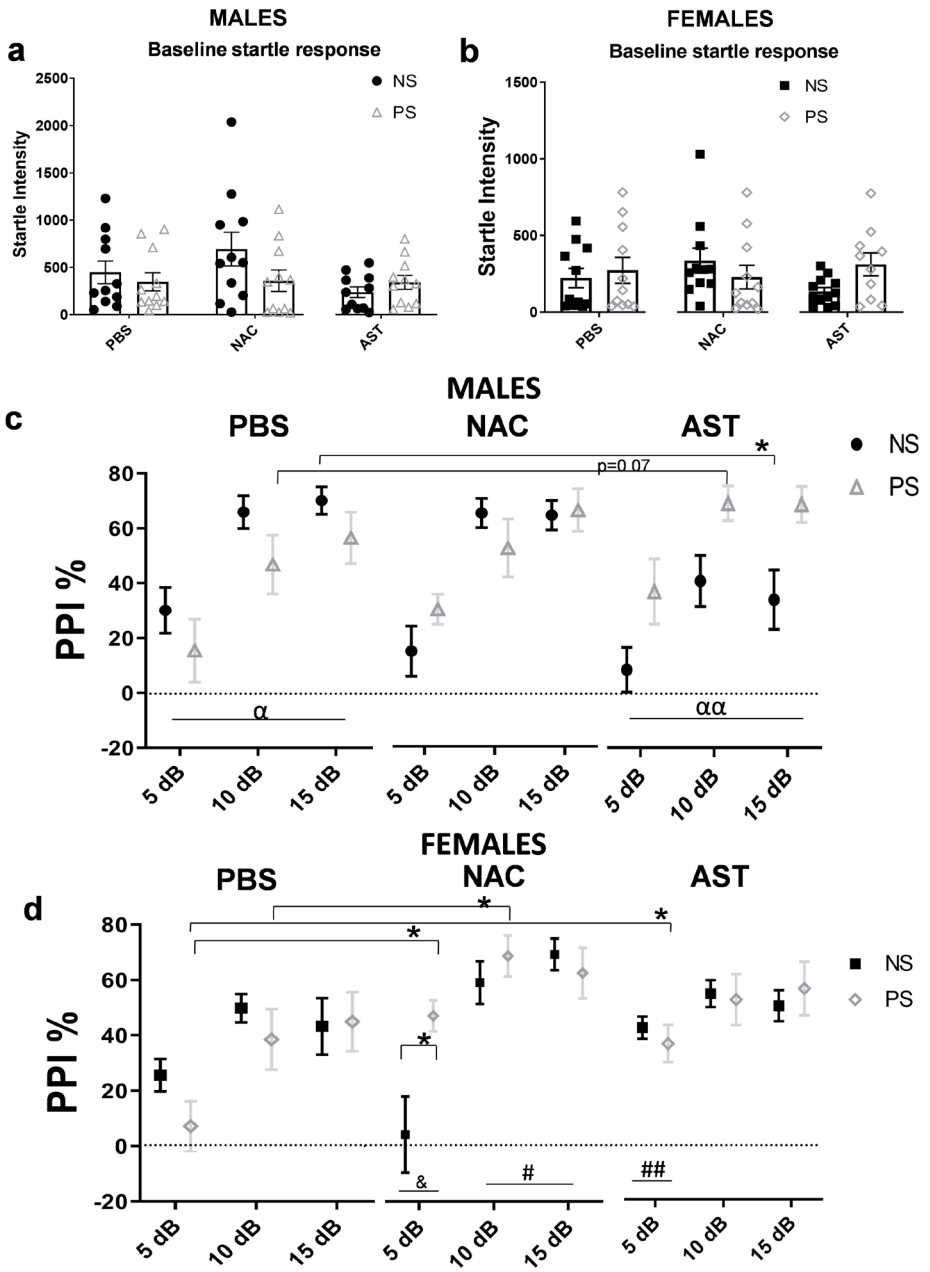


**Figure 6**. **Prenatal stress altered sensorimotor gating in male offspring, and a trend for AST to prevent this was found**. No differences in the baseline startle response were observed in (**a**) male offspring or (**b**) female offspring groups. (**c**) prenatally-stressed (PS) PBS male mice showed lower PPI across all three decibel (dB) levels (α $p<0.05$ by two-way ANOVA). Non-stressed (NS) AST male mice showed lower PPI across the three dB levels compared to PS AST mice (αα $p<0.01$ by two-way ANOVA). At the 10 dB level, PS AST showed trend higher sensorimotor gating compared to PS PBS male mice (*post-hoc*, trend sig., $p=0.07$). (**d**) An interaction between NAC and prenatal stress at 5 dB was found and suggests that NAC on its own is decreased 5 dB PPI in female mice (& $p<0.05$ by two-way ANOVA). At the 5 dB level, PS NAC and PS AST showed improvements in sensorimotor gating compared to PS PBS female mice (*post-hoc*, * $p<0.05$, respectively) and a main effect of AST demonstrated its general improvement of PPI in female mice (## p<0.001 by two-way ANOVA). At the 10 dB and 15 dB levels, a main effect of NAC demonstrated its improvement of PPI (# p<0.05 by two-way ANOVA for 10 dB and 15 dB, *post-hoc* for 10 dB, * $p<0.05$). Means and Standard errors of the mean are displayed.

**Table 1. Summary of Effects of Prenatal Stress and Maternal Antioxidant in CD1 Mice**

| Outcome | Prenatal stress effect? | Antioxidant prevention? | Other effects |
|---|---|---|---|
| **Cortical Microglia** | M: ↓lowly, ↑highly ramified morphologies | M: NAC prevented effects on lowly ramified microglia | M: N/A |
| | F: ↓lowly, ↑highly ramified morphologies | F: NAC & AST prevented effects on lowly, highly ramified microglia; AST prevented effects on amoeboid microglia | F: N/A |
| **Interneurons** | M: ↑mPFC PV/GAD67 ratio ↓Hippocampal GAD67 | M: NAC prevented mPFC effect, AST prevented Hippocampal effect | M: AST ↓mPFC PV/GAD67 in all males, Hippocampal GAD67 correlated with OF Ctr time |
| | F: ↓mPFC PV & PV/GAD67 ↓Hippocampal PV/GAD67 ratios | F: AST prevented both mPFC effects, NAC prevented mPFC PV effect and Hippocampal effect | F: N/A |
| **Anxiety-like Behavior** | M: ↓open/closed ratio on EPM | M: no | M: NAC & AST ↑OF Ctr time in all males, Hippocampal GAD67 correlated with OF Ctr time |
| | F: ↓open/closed ratio on EPM | F: no | F: N/A |
| **Locomotion** | M: no effect | M: N/A | M: N/A |
| | F: no effect | F: N/A | F: NAC ↑distance in NS females |
| **Social Behavior** | M: no effect | M: N/A | M: N/A |
| | F: ↓sociability | F: no | F: N/A |
| **Sensori-motor Gating** | M: ↓PPI (5, 10, 15 dB) | M: AST trend prevented ↓ PPI | M: AST ↑PPI in PS male, AST ↓PPI in NS males |
| | F: no effect | F: N/A | F: NAC & AST ↑PPI in PS females |

M = males, F = females, NAC = N-acetylcysteine, AST = astaxanthin, mFC = medial frontal cortex, PV = parvalbumin+ cells, GAD67 = GAD67GFP+ cells, EPM = elevated plus maze, OF Ctr = center of the open field, PPI = pre-pulse inhibition, dB = decibels, PS = prenatally-stressed, NS = non-stressed

spite some neurobiological protection from antioxidants. These data suggest a few possible scenarios concerning prenatal stress mechanisms which are not mutually exclusive: one possibility is the cellular neurobiology assessed here may have relevance for other behavioral outcomes not assessed here and vice-versa; a related potential implication is break-through levels of oxidative stress may have exceeded the capacity of antioxidant effects resulting in neurobiological impacts other than those studied here and demonstrated effects on behavior; and/or other co-occurring mechanisms of prenatal stress effects (ie, inflammation and/or glucocorticoid actions) may have initiated changes in offspring responsible for the behaviors assessed here. Further investigations of the neurobiological underpinnings of disrupted behavior after prenatal stress will be important, particularly in relation to the role of prenatal processes in male offspring targeted by N-acetylcysteine in establishing sensorimotor gating functions of the brain.

The role of oxidative stress in prenatal stress outcomes in this study was most clear for cortical microglia changes, converging with many lines of research showing the importance of oxidative stress for microglia development [35,37]. Prenatal stress results in oxidative stress in both placenta and offspring brain [21,24,69,70] which are both theoretically targets for maternally-administered NAC and AST; NAC crosses the placenta [71] and AST's properties suggest it has placenta transfer. Impacts of pre-

natal stress on interneuron populations were not consistently prevented by both antioxidants, but the efficacy of NAC and AST for protecting specific outcomes suggests that distinct, non-redox aspects of these antioxidants may have played a role, including the NMDA receptor modulatory effects of NAC and the anti-inflammatory and mitochondrial functional impacts of AST which are important pathways relevant to the development of neuropsychiatric disorders [57,58,64,67,68]. Either redox and non-redox mechanisms may also have accounted for the impacts of NAC and AST on neurobiology and behavior of control (non-stressed) offspring (Table 1).

The impacts of prenatal stress on cortical microglia and their prevention by maternal antioxidants suggests that *in utero* oxidative stress programs either the embryonic microglia and/or their milieu to result in adult microglia morphology with less activated morphologies but more bushy morphologies which may reflect more primed cells. Interestingly, this diverges from the traditionally-conceptualized paradigm of oxidative stress inducing more active microglia [37], but like others who have studied these phenomena across different developmental stages [38], this may demonstrate the complexity of microglia development and their multiple forms. Our findings align with reports of more primed microglia in neuropsychiatric disorders [33,34] which may be sensitive to additional insults. Primed microglia have the capacity more quickly and to a greater extent to activate and alter synapses and other components of their milieu critical for neuronal control of behavior [72]. Primed microglia may underlie behavioral changes in settings of acute stress or insults, which is a hallmark of many neuropsychiatric disorders and could be assessed in future studies for dependence on prenatal oxidative stress.

The impacts of prenatal stress on cortical and hippocampal interneurons in general demonstrates the vulnerability of the parvalbumin subtype to pro-oxidant states, but also the potential for pro-oxidant disruption of development and/or function of other GABAergic interneuron subtypes as reflected in the total GAD67+ density. While we found no correlations of parvalbumin populations with behavior, reduced parvalbumin cell density and proportion of total GABAergic cells in female offspring aligns with findings in patients with ASD; hypofunction of these subsets of neurons would be predicted to disrupt multiple ASD-relevant behaviors through altered sensory tuning and circuit oscillation, impacting sensory discrimination and perceptual learning [73]. Correlations between cortical and hippocampal total GAD67+ cell density and anxiety-like behavior in male offspring converges with other data suggesting their critical role in behavioral inhibition through impaired fidelity of cortical information processing or increased suppression of limbic responses [47,74]. By examining these and other interneuron subtypes in future studies, specific neuropeptides may be identified as targets for neuropsychiatric treatments.

We examined both males and females for the effects of prenatal stress on neurobiology and behavior and how antioxidant administration prevented these. Our study's conclusions and its statistical differences were limited by the small sample size. However, some outcomes were consistent across males and females, suggesting *in utero* pathways of effect not dependent on sexual dimorphism of the placenta or early brain development. The increase in cortical microglia ramification with prenatal stress that was prevented by NAC or AST in females was similar to the significant microglia change in males: the decrease in lowly ramified microglia with prenatal stress and the significant impact of NAC and AST on lowly ramified microglia (Figure 1c). Male and female similarity of prenatal stress effect was also found on anxiety-like behavior, although was not prevented by antioxidants (Figure 2a-d). However, prenatal stress effects on cortical and hippocampal interneurons diverged with offspring sex, with PV+/GAD67+ ratio decreased in females (mainly due to decreased PV+ density, prevented by NAC and AST, Figure 2f) and increased in males (mainly due to decreased GAD67+ density, prevented only by AST, Figure 3c). This potential for the same prenatal antioxidant to rescue distinct impacts of prenatal stress on interneurons—either PV subtype differentiation or total GABAergic population—suggests that common elements across males and females in interneuron development may interact with later sex-specific divergent paths. Effects of prenatal stress on sociability were only found in females (Figure 4f) and on sensorimotor gating (prevented by AST, Figure 5c) were only found in males. Sex differences are common in prenatal stress studies, although we did not find a predominant male vulnerability for these outcomes, as in other studies [75,76]. Sex differences in neurobehavioral outcomes in prenatally-stressed adult offspring may originate from effects of prenatal stress interacting with pubertal sexual dimorphism or from earlier sex differences—one increasingly important origin of neurodevelopmental abnormalities and their sex-specificity are placenta changes [69,77,78] which represent a more accessible target for preventive intervention targeting oxidative stress and other effects. Targeting of only maternal and placental oxidative stress with the nanoparticle-bound antioxidant MitoQ has demonstrated prevention of sex-specific interneuron and anxiety-like behavior changes after prenatal stress [69].

In conclusion, we observed that prenatal stress effects on cortical microglia, cortical and hippocampal interneurons, and sensorimotor gating were preventable with maternal antioxidants but effects on anxiety-like behavior and sociability were not. Interestingly, early exposure to antioxidants *in utero* prevented changes in

adult offspring cortex microglia to atypical morphologies after prenatal stress including a substantial increase in highly ramified cells suggestive of a primed population. Finally, sex differences were clear in cortical and hippocampal interneuron changes after prenatal stress; despite this, divergent male and female neurobiological change may result from some common mechanisms due to the ability of antioxidants to prevent effects across sexes. The relationship of these neurobiological changes to neuropsychiatric vulnerability will be an important area of future investigation.

**Acknowledgments:** HES and JL-BD were supported by Roy J. Carver Charitable Trust and Nellie Ball Trust research grants, NIH R01 MH122485-01, Environmental Health Science Research Center career development award (NIH P30 ES005605), and University of Iowa (UI) Graduate College Dean's Fellowship. HE was supported by UI Carver College of Medicine Summer Research Fellowship. SLS was supported by UI Biomedical Scholars Summer Undergraduate Research Program.

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