The role of redox dysregulation in the effects of prenatal stress on the embryonic and adult mouse brain

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THE ROLE OF REDOX DYSREGULATION IN THE EFFECTS OF PRENATAL STRESS ON THE EMBRYONIC AND ADULT MOUSE BRAIN

by

Jada Leanne-Bittle Davis

A thesis submitted in partial fulfillment of the requirements for the Doctor of Philosophy degree in Neuroscience in the Graduate College of The University of Iowa

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ABSTRACT

Maternal stress during pregnancy is associated with increased risk of psychiatric disorders in offspring, but embryonic brain mechanisms disrupted by prenatal stress are not fully understood. Our lab has shown that prenatal stress delays inhibitory neural progenitor migration. Here, we investigated redox dysregulation as a mechanism for embryonic cortical interneuron migration delay, utilizing direct manipulation of pro- and anti-oxidants and a mouse model of maternal repetitive restraint stress starting on embryonic day 12. Time-lapse, live-imaging of migrating GABAergic interneurons showed that normal tangential migration of inhibitory progenitor cells was disrupted by the pro-oxidant, hydrogen peroxide. Interneuron migration was also delayed by in utero intracerebroventricular rotenone. Prenatal stress altered glutathione levels and induced changes in both activity of antioxidant enzymes and expression of redox-related genes in the embryonic forebrain. Assessment of dihydroethidium (DHE) fluorescence after prenatal stress in ganglionic eminence, the source of migrating interneurons, showed increased levels of DHE oxidation. Maternal antioxidants (N-acetylcysteine and astaxanthin) normalized levels of DHE oxidation in ganglionic eminence, and ameliorated the migration delay caused by prenatal stress.

In adult male offspring, prenatally-stressed mice exhibited anxiety-like behavior on the elevated plus maze, impaired motor learning on the rotarod, cognitive flexibility on the water T-maze task, and deficits in sensorimotor gating in the pre-pulse inhibition task. Prenatally-stressed adult female offspring showed anxiety-like behavior, deficits in sociability and impaired motor learning. Maternal antioxidants prevented anxiety-like behaviors and improved sensorimotor gating in both sexes, and improved habitual learning and cognitive flexibility in adult female mice. Lastly, prenatal stress led to increases in PV+/GAD67+ cell ratios in mFC in
male mice, but decreases in female mice, and antioxidant treatments eliminated those differences. Hippocampal GAD67+ cell densities were reduced by prenatal stress and restored by AST in male mice, and PV+/GAD67+ cell ratio was reduced by prenatal stress and partially restored by NAC in female mice. Lastly, GAD67+ cell densities across regions correlated significantly with anxiety-like behavior in both male and female mice and social behavior in female mice. Through convergent redox manipulations, delayed interneuron migration after prenatal stress was found to critically involve redox dysregulation. Redox biology during prenatal periods may be a target for protecting brain development.
PUBLIC ABSTRACT

Stress experienced by the mother during her pregnancy can affect her baby’s brain development. Prenatal stress (PS) is associated with an increased risk of the child developing a psychiatric disease, such as schizophrenia and anxiety. Because we cannot prevent stressful life events, my goal is to understand the cellular changes that take place in utero with the ultimate goal of protecting the embryonic brain during these stressful events and throughout the rest of development. Our lab has shown in mice that PS delays the migration of inhibitory neurons in the embryonic brain during development. Here, we found increased levels of oxidative stress in the embryonic brain and neuron migration delays similar to those we observed after PS. We also found that maternal treatments of antioxidants rescued the delays and prevented oxidative stress in the embryonic brain.

Our lab has shown in mice that PS can have lasting consequences into adulthood. We were interested in whether oxidative stress during embryonic brain development (similar to PS) could have the same effects. In male offspring mice, PS mice exhibited anxiety-like behavior, impaired motor learning, better cognitive flexibility, and problems dampening their startle reflex in response to an acoustic sound. PS female offspring showed anxiety-like behavior, deficits in sociability and impaired motor learning. Maternal antioxidants prevented anxiety-like behaviors and improved sensorimotor gating in both sexes, and improved habit learning and cognitive flexibility in female offspring. Lastly, in the prefrontal cortex and hippocampus, maternal antioxidant treatment prevented an imbalance of inhibitory neurons and their subtypes.
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CHAPTER 1. INTRODUCTION

Stress experienced by the mother during pregnancy can significantly affect embryonic development and fetal physiology (Wadhwa, 2005). Prenatal stress is associated with an increased risk for neuropsychiatric illness, such as attention-deficit hyperactivity disorder (ADHD) (Huot et al., 2004) and schizophrenia (King and Laplante, 2005; Bale, 2009), as well as childhood behavioral, physiological, and emotional problems in offspring (O’Connor et al., 2003; Markham and Koenig, 2011; Monk et al., 2012). During embryonic development, environmental factors such as maternal stress are associated with an increased risk of psychopathology in offspring and these factors are known to alter mature brain functioning in many different ways (Weinstock, 2008). Alterations to the developing brain during prenatal stress must have enduring consequences in order to explain the development of neuropsychiatric illness during adolescence and adulthood, many years later. The cellular and molecular alterations in the fetal brain that occur during prenatal stress are therefore a significant part of understanding psychopathology.

Modeling the transmission of psychological stress from mother to biological changes in the fetus that lead to the risk of neuropsychiatric disease in her offspring is difficult to achieve in an ethical manner. Experimental data shows that restraint stress in rodents can replicate some of the abnormalities in offspring behavior observed in humans (for a review, please see (Weinstock, 2017) making it a useful tool in our modeling efforts. Similar to humans, when a stressor is experienced by a rodent, the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system are activated. Repeated triggering of the HPA axis, or the physiological response of “fight or flight” mode, can become maladaptive, however, when the perception of a
threat or stressor is inconsistent with the actual magnitude and realized present danger. On the other hand, habituation to the stressor leaves the HPA response weaker after repeated exposures to that stressor (Nesse et al., 2007). Habituation of the HPA response in most situations is adaptive; however, the HPA response can be maladaptive if the stressor still presents some risk of danger. In this case, the habituation to the stressor leaves the rodent vulnerable to the dangers of the stressor. In attempting to model psychological stress in rodents, the stressor is designed to cause distress and evoke adaptive physiological response and release of hormones in the mother without causing tissue damage to the fetus (Graignic-Philippe et al., 2014). Utilizing a restraint stress model with rodents is the most ethical way to study the phenomenon of maternal psychological stress during pregnancy and the unfavorable life-long outcomes on child development. Animal models are essential to our understanding of brain development, and research using rodents, non-human primates, and other vertebrates suggests that prenatal stress impacts development of GABAergic neural circuitry in the mature brain and adult psychopathology (Matrisciano et al., 2013). The ultimate goal is to understand the mechanisms by which prenatal stress affects childhood and adult psychopathology and to develop better treatments for those at risk for neuropsychiatric disease.

**GABAergic systems and psychiatric illness**

There are important relationships between the inhibitory neural circuitry of the forebrain and mental illness, including schizophrenia and autism (Benes and Berretta, 2001; Hashimoto et al., 2008; Yip et al., 2008; Curley et al., 2011). Disruption of GABAergic forebrain systems during embryonic development can have significant, enduring consequences for neural function in adolescence and adulthood (Powell et al., 2003; Levitt et al., 2004; Fu et al., 2012). The
Effects of prenatal stress on the brain have become a major focus of the investigation into potential mechanisms by which the functioning of GABAergic systems are affected by the environment. Inhibitory cortical interneurons are of particular significance because of their role in cognition (Fishell and Rudy, 2011a) and links between atypical GABAergic system functioning and the psychopathology of schizophrenia, autism, and anxiety disorders (for a comprehensive review see (Fine et al., 2014)).

The effects of prenatal stress on the embryonic brain have revealed potential mechanisms through which the functioning of cortical GABAergic systems are affected by early environmental stressors. From their birthplace in the ganglionic eminence, GABAergic progenitor cells tangentially migrate into the developing neocortex, where they establish inhibitory neural connections to form functional groups such as cortical columns and limbic-cortical loops (Wonders and Anderson, 2006). In early postnatal brain development, young GABAergic neurons are known to regulate and ensure the proper functioning of the cerebral cortex during critical periods of maturation (Powell et al., 2011). In the mature brain, they act as modulators of excitatory networks and alter excitatory synapses in principal neurons (Fishell and Rudy, 2011b). GABAergic neurons are also involved in the generation of gamma oscillations, which regulate neural synchrony and temporal coordination in the mature brain. Gamma-oscillatory activity is essential for temporal structure of information processing, i.e., attention, perception, working memory, and behavioral regulation (Powell et al., 2011).

The pathophysiology of neuropsychiatric disorders, such as schizophrenia, is complex and involves many different cortical and subcortical networks. One subtype of inhibitory cortical neuron, parvalbumin (PV), is a calcium-binding protein that is expressed by a population of interneurons found throughout the cerebral cortex. These fast-spiking interneurons are
essential for temporal structure of information processing and abnormalities in PV interneurons may underlie the cognitive disturbance association with schizophrenia (Powell et al., 2011; Lewis et al., 2012). Schizophrenia is associated with a loss of GABAergic neurons in the hippocampus and neocortex, and neurons expressing PV and somatostatin are known to display altered functioning in patients with schizophrenia. Postmortem studies of patients with schizophrenia also reveal an increase in GABA_A receptor activity in hippocampal CA3 neurons, but no difference in benzodiazepine receptor binding on either pyramidal or nonpyramidal neurons (Benes et al., 1997). These alterations are thought to be related to abnormal oscillatory rhythms and increased basal metabolic activity in the hippocampal formation of patients with schizophrenia. Alterations in the GABA system may be associated with early life stress and not related to a susceptibility gene for schizophrenia, as previously hypothesized (Benes and Berretta, 2001).

Patients with schizophrenia and schizoaffective disorder were found to have lower GAD67 (glutamic acid decarboxylase 67) protein levels in the dorsolateral prefrontal cortex relative to healthy, comparison subjects (Curley et al., 2011; Lewis et al., 2012). GAD67 is an important enzyme involved in the synthesis of inhibitory neurotransmitter, GABA, which plays a key role in synaptogenesis during development. Deletion of the gene encoding GAD67 results in a 90% reduction in brain GABA levels and is embryonically lethal in mice (Asada et al., 1997). Research has consistently shown substantial dysregulation of GAD67 mRNA expression in patients with schizophrenia, but the mechanism underlying the decreased levels of GAD67 protein has yet to be identified (Woo et al., 2004).

Using animal behavioral models, researchers have been able to examine long-term changes in brain function after prenatal stress. Prenatal stress-exposed rat pups have
significantly increased distress vocalizations, decreased social learning and an impaired responsiveness in social relationships (Harmon et al., 2009; Laloux et al., 2012). Neurochemical and endocrinological alterations are associated with anxiety-like behavior in male rat pups exposed to prenatal restraint stress. Compared to control rat pups of non-stressed mothers at postnatal day 14 (P14), prenatally-stressed rat pups do not show the plasma peak in leptin levels (Laloux et al., 2012)—a hormone known for its role in feeding behavior and mediating important cellular events such as neurogenesis, axon growth, and synaptogenesis (Bouret, 2010).

Moreover, prenatally-stressed adolescent pups show reduced expression of the γ2 subunit of GABA_A receptors and an increased expression of mGlu5 receptors in the amygdala, and reduced expression of mGlu5 and mGlu2/3 receptors in the hippocampus. These neurochemical and endocrinological changes correlated with anxiety-like behaviors, such as ultrasonic vocalizations, in prenatally-stressed rat pups (Laloux et al., 2012).

**Prenatal stress and GABAergic cell migration**

Many studies have demonstrated that prenatal stress has a variety of deleterious effects on the development of inhibitory neuron systems in the embryonic and neonatal brain (Fine et al., 2014). Inhibitory neuron progenitor cells migrate a great distance from their subcortical birthplace in the ganglionic eminence before taking up permanent residency in the cerebral cortex (Polleux, 2002). Transcription factors that regulate GABAergic interneuron migration are significantly changed by prenatal stress (Stevens et al., 2013) and maternal immune activation (MIA) (Oskvig et al., 2012). Prenatal stress and the maternal immune factor, interleukin 6 (IL-6), are also known to decrease overall migration of cortical interneuron progenitors (Gumusoglu et al., 2017b) and decrease the density of GABAergic progenitors in the embryonic cortical plate.
(Stevens et al., 2013). However, up-stream mechanisms by which prenatal stress leads to GABAergic progenitor changes have yet to be elucidated.

Due to the importance of GABAergic network activity for GABA neuron development (Baho and Di Cristo, 2012), delays in the migration of the developing GABAergic cells may cause the maturation of these cell populations to be prolonged or impeded (Powell et al., 2003). Interruptions in the maturation of GABAergic cells that occur in early development could persist and may be responsible for abnormal GABAergic system functioning in adolescence and adulthood (Stevens et al., 2013). Cell autonomous transcription factors known to regulate GABAergic cortical interneuron migration, such as Distal-less 2 (DLX2), NK2 homeobox 1 (NKX2.1), and their downstream target Erb-B2 Receptor Tyrosine Kinase 4 (ERBB4), are significantly changed by prenatal stress (Stevens et al., 2013). Moreover, extracellular cues are also critical for GABAergic progenitor migration, including motogens, chemoattractants, and neurotransmitters (Corbin and Butt, 2011). Therefore, disruptions of both cell-autonomous and non-cell-autonomous processes by prenatal stress may influence the migration of GABAergic cells. Other studies modeling GABAergic cell migration during embryonic brain development reveal long-lasting effects in cortical inhibitory neuron populations, especially the PV subtype (Flames et al., 2004; Meechan et al., 2012; McCarthy et al., 2014).

GABAergic interneuron progenitor migration into the developing neocortex is delayed by prenatal stress in mice (Stevens et al., 2013). In normal development, after this migration, total GABAergic cell numbers in the brain peak during adolescence. Prenatal stress also alters the timing of the peak trajectory of total GABAergic cell totals in the medial frontal cortex (mFC) and hippocampus. A correlation of poor performance on behavioral tasks and altered total GABAergic cell totals were observed in adult male offspring mice. Prenatally-stressed mice
showed enhanced anxiety-like behavior on the elevated plus maze, reduced center time in the open field task, and a lack of social preference on the three-chambered task (Lussier and Stevens, 2016). While the researchers found that prenatal stress lead to a delay in the maturation of GABAergic cells in the mFC and hippocampus during adolescence, the total population of GABAergic cells normalized by adulthood (Lussier and Stevens, 2016). Taken together, these studies suggest that prenatally-stress-induced disruptions in tangential migration of GABAergic neural progenitor cells may have consequential effects many weeks after the prenatal insult.

**GABAergic neural systems and redox dysregulation**

There are a number of cellular and molecular mechanisms that direct cortical interneuron migration from the subpallium to the developing cortex. Extracellular cues, such as brain-derived neurotrophic factor (BDNF), hepatocyte growth factor (HGF), and other motogenic molecules, are crucial to migration (Brunstrom et al., 1997; Powell et al., 2001; Polleux, 2002). Chemoattractants, such as the Neuregulin (Nrg) family of epidermal growth factor (EGF)-like proteins, can act as short-range and long-range attractants aiding the tangential migration of interneurons (Flames et al., 2004; Bartolini et al., 2017). In contrast, chemorepulsive signals repel cortical interneurons from remaining in the ventral forebrain or entering other developing regions of the brain. For example, semaphorin/neuropilin signaling repels interneurons destined for the cortex from entering the striatum (Marín et al., 2001). Cell autonomous components of interneurons such as CXCR4, the chemokine receptor for the chemoattractant, SDF-1, also guide interneurons within the tangential migratory streams (Sanchez-Alcaniz et al., 2011). Genetic and syndromic risk for psychiatric illness influences these mediators and cortical interneuron development (Meechan et al., 2012; Abbah and Juliano, 2014). The role of maternal
physiological disruptions such as prenatal stress in affecting these mechanisms has not been explored.

GABAergic interneuron migration is also susceptible to changes to oxidative phosphorylation (Lin-Hendel et al., 2016). Both genetic disruption of mitochondrial OXPHOS in mice lacking adenine nucleotide transferase 1 (Ant1) and pharmacological inhibition of OXPHOS are associated with alterations of interneuron migratory morphology, abnormal migration behavior, and mispositioning of the centrosomes. The migration patterns of cortical interneurons with dysfunctional mitochondria exhibited more frequent and aberrant directional changes and slower migratory rates (Lin-Hendel et al., 2016). Mitochondrial disruption also significantly contributes to the generation of reactive oxygen species (ROS) (Burton and Jauniaux, 2011), adding to the reactive forms of oxygen produced during normal metabolism (Guerin et al., 2003) from the one electron reductions of O\(_2\) from mitochondrial electron transport chains. The embryonic brain is particularly vulnerable to ROS changes and redox dysregulation because of its low antioxidant capacity (Wells and Winn, 1996; Wells et al., 1997; Wells et al., 2005; Shim and Kim, 2013), its high oxygen utilization, its abundance of oxidizable polyunsaturated fatty acids, its high content of redox-active metals (Cu and Fe) capable of catalyzing free radical formation, and its large proportion of sensitive immature cells (Ikonomidou and Kaindl, 2010). Given the great distance tangentially migrating GABAergic progenitor cells must cover to reach their final destination, they have higher oxygen utilization making them particularly vulnerable to redox dysregulation (Narasimhaiah et al., 2005). This is bolstered by evidence that tangentially migrating progenitor cells are uniquely susceptible to disruptions to oxidative phosphorylation in ways radially migrating neurons are not (Lin-Hendel
et al., 2016). Therefore, ROS and redox balance likely plays a role in disruptions of GABAergic progenitor migration into the developing neocortex and overall embryonic brain development.

**Redox dysregulation and fetal programming**

There are multiple links between redox regulation and the development of inhibitory neural systems (Marín, 2012; Hsieh et al., 2017). Redox dysregulation results from disruptions to the balance of reductive and oxidative molecules and processes of cellular metabolism. Sizable, sustained redox dysregulation may occur when ROS overwhelm endogenous antioxidants (or result from impaired antioxidants) and may contribute to multiple disease states (Peuchant et al., 2004; Cambonie et al., 2007b; Derks et al., 2010a; Ghulmiyyah et al., 2011; Ziech et al., 2011; Pinney and Simmons, 2012). Neuropsychiatric disorders have been specifically linked to redox dysregulation, including schizophrenia (reviewed in (Do et al., 2009)), epilepsy (Chevallier et al., 2014) and autism spectrum disorder (ASD) (Rossignol and Frye, 2012). Inhibitory circuits are implicated in these disorders (Marín, 2012) and fast-spiking Parvalbumin interneurons are particularly sensitive to redox dysregulation (Do et al., 2009). Research conducted with the purpose of understanding schizophrenia has identified developmental redox dysregulation as constituting a “final common pathway” or “hub” where genetic impairments of glutathione synthesis and environmental vulnerability factors generate oxidative stress (Do et al., 2009). Using experimental models, they identified redox dysregulation as mediating hypoactive NMDA receptors, abnormalities in fast-spiking Parvalbumin interneurons, and deficits in myelination (Do et al., 2009), all contributing to impairments to inhibitory neural connectivity and synchronization that is characteristic of the pathology of schizophrenia.
Redox dysregulation is one of the potential mechanisms by which prenatal stress affects cellular changes in the fetal brain, making it a rich avenue of exploration. But one inescapable problem with studying oxidative stress generally is the issue of an imbalance in the pro-oxidant-antioxidant homeostasis, which suggests that problems could arise from perturbation on either side of the equilibrium. A delicate balance between the reductive and oxidative states is crucial for normal cellular metabolism and a disruption in the balance is termed “redox dysregulation” (of which oxidative stress is a component). Consequently, there is a biological goldilocks zone of relative levels of ROS wherein excess levels of ROS are equally as detrimental as insufficient levels of ROS at critical periods of development (Castagné et al., 1999). An additional problem is attempting to study redox dysregulation during embryonic development since ROS are vital to normal embryonic development through cellular signaling and regulation of cellular fate (for an extensive review on the subject, please see Dennery (2007)). ROS are capable of being created and degraded quickly through enzymatic reactions making them ideal second messengers. In their role as second messengers, they act by regulating important transcription factors, such as activator protein (AP-1) and nuclear respiratory factor 1 (NRF1), which are vital to normal embryonic development. AP-1 controls DNA repair, apoptosis, and antioxidant genes during development (Gómez del Arco et al., 1997) and NRF1 controls apoptosis, redox balance and antioxidant genes during development (Chen et al., 2003).

During the course of ATP production in mitochondria through oxidative phosphorylation (OXPHOS), free radicals, including ROS and reactive nitrogen species (RNS), are generated as a by-product from the one electron reductions of $O_2$ from mitochondrial electron transport chains. Free radicals are characterized as any atom or molecule possessing one or more unpaired electrons in the outer shell. While some free radicals are notably less reactive than others, most
free radicals relevant to biological function are highly reactive and can cause chain reactions forming other highly reactive molecules. The superoxide radical (O$_2^-$) is the most ubiquitous free radical in the body and is generated in the mitochondria of cells. O$_2^-$ is detoxified by the enzyme superoxide dismutase (SOD), which catalyzes the dismutation of the molecule into hydrogen peroxide (H$_2$O$_2$). H$_2$O$_2$ is less reactive than O$_2^-$, so it is not considered a free radical in the traditional sense. It is, however, responsible for the generation and detoxification of free radicals, so it does fall under the category of an ROS molecule. H$_2$O$_2$ and O$_2^-$, in the presence of free iron ions, can form the more dangerous and highly reactive, hydroxyl ion. This reaction is catalyzed by iron during the Fenton reaction and hydroxyl ions are known to react with any biological molecule in their immediate vicinity and cause damage. O$_2^-$ can also interact with nitric oxide to form the highly reactive ion, peroxynitrite. Fortunately, enzymatic and non-enzymatic defenses, in the form of antioxidants including the enzyme catalase and nutrient Vitamin C, detoxify H$_2$O$_2$ and O$_2^-$ to the harmless molecule, H$_2$O (Burton and Jauniaux, 2011).

ROS participate in normal physiological roles such as cell differentiation and cell proliferation, but in excess, can contribute to the pathogenesis of disease by impairing modulation of signal transduction and/or by causing oxidative damage to cellular macromolecules including proteins, lipids, and DNA. Several genes vital to oxygen detection, cell differentiation, and cell proliferation, such as HIF1A, CREB1, and NFKB1, have been shown to be induced for transcription by ROS (Castagné et al., 1999; Schafer and Buettner, 2001). ROS molecules can also directly modify histones and alter DNA methylation by interacting with DNA base pairs, causing both genetic and epigenetic changes during development. The influence of ROS on DNA methylation has become an important area of study because of its potential to disrupt normal gene expression. Furthermore, ROS-generating enzymes, such as NADPH-
oxidase 2 (NOX2), have been shown to modify the mRNA levels of important neuronal and neural stem/progenitor markers such as Nestin, Dcx, Bdnf, and Otx2, in neurogenic regions of the mouse cortex and are responsible for maintaining the neural progenitor cell pool (Nayernia et al., 2017). There is strong evidence that ROS play a crucial role in embryonic neurogenesis in part by regulating stem cell homeostasis and cell differentiation (Yoneyama et al., 2010; Dickinson et al., 2011; Le Belle et al., 2011).

As mentioned above, normal embryonic development occurs in a relatively low-oxygen environment. Under normal conditions in utero, there is very low arterial oxygen tension (PaO2) with about 32 mm Hg in the umbilical vein and 22 mm Hg in the descending aorta (Émond et al., 1993) compared to 75-100 mm Hg arterial pressure in adult mammals. However, as the embryo continues to develop, the placenta matures and increases its oxygen transfer to the developing fetus. Increased levels of oxygen from the placenta and adequate nutrients from the intrauterine environment are essential for sustaining the increased metabolic demand of the growing fetus. This rapid growth phase makes the fetus vulnerable to the effects of hypoxia which may play a role in fetal programming—the notion that any stimulus or insult during sensitive periods of development can alter the development of the fetus with permanent effects on the child that carry into adulthood (Kwon and Kim, 2017). Utilizing a chick embryo model, researchers discovered that hypoxia, independent of maternal nutrition during development, can result in restricted fetal growth (Giussani et al., 2007). Intrauterine stress and fetal hypoxia have been widely studied using animal models. Among studies of hypoxia-induced fetal growth restrictions, chronic fetal hypoxia has been found to increase neuronal nitric oxide synthase (Dong et al., 2011), decrease the antioxidant state in the brain, and shift the balance to a more pro-oxidant state in the brain of guinea pigs (Guo et al., 2010).
While fetal hypoxia-induced oxidative stress is known to be involved in the pathogenesis of fetal growth restriction, studies on preterm infants have shed light on the susceptibility of the brain to \textit{hyperoxia}-induced oxidative stress (Back et al., 1998; Baud et al., 2004b; Baud et al., 2004a; Felderhoff-Mueser et al., 2004; Gerstner et al., 2008). Research has shown preterm infants are particularly vulnerable to oxidative stress-induced damage to the brain due to the “relative hyperoxia”, or unphysiologically high levels of oxygen, outside the \textit{in utero} environment. It is believed that the developing brain is susceptible to hyperoxia for the same reasons it is susceptible to hypoxia: both physiological states generate large quantities of free radicals and often suppress antioxidants in an organ that is already not fully equipped to combat the ROS-mediated assault (Ikonomidou and Kaindl, 2010). However, in oviparous animals, maternally derived yolk antioxidants successfully buffered the developing embryonic tissues from lipid peroxidation following an external oxidative challenge of hyperoxia (Watson et al., 2018). Elucidating the mechanisms that connect hypoxia- and hyperoxia-induced ROS generation and genetic and epigenetic changes during development is important to understanding the long-term consequences of cell redox disturbances and fetal programming.

Epidemiological evidence supports the role of redox dysregulation in fetal programming with regards to high scores on oxidative stress indices and low birth weight in association with preeclampsia (Roberts and Lain, 2002). Obesity during pregnancy may affect maternal and fetal development through changes in ROS biomarkers in humans. Newborns of obese mothers have lower levels of plasma total antioxidant activity (ORAC) and increased levels of hydroperoxides and antioxidant enzyme activities (catalase, SOD, glutathione reductase and peroxidase) as compared to control newborns (Malt-Boudilmi, 2010). Another study characterized the architectural and physiological alternations in brains of children and adults with ASD and found
that the pathophysiologic mechanisms likely originate during fetal development and are associated with redox dysregulation (Walker et al., 2015). These studies provide evidence that ROS changes during prenatal development could lead to long-lasting changes in the brain of offspring postnatally.

**Prenatal and postnatal antioxidants as manipulators of redox regulation**

Prenatal stress has been known to induce redox dysregulation and behavioral deficits in offspring mice. Studies have found that prenatal stress causes a decrease in total antioxidant activity, SOD activity, reduced glutathione (GSH) levels, and glutathione peroxidase (GPx) in the postnatal day (P24) mouse brain. Malondialdehyde (MDA), a measure of lipid peroxidation, in the juvenile mouse brain also increases as the result of prenatal stress (Bernhardt et al., 2017). Treatment with N-acetyl cysteine (NAC), a glutathione precursor and powerful antioxidant, during the prenatal period as an antioxidant supplement reverses brain redox changes after prenatal stress in adult mice (Bernhardt et al., 2017) and reverses effects of prenatal stress on embryonic microglia (Bittle and Stevens, 2018). However, what remains to be examined is the efficacy of maternal antioxidant treatments during prenatal stress altering the redox balance in the embryonic brain and the behavioral outcomes of adult offspring.

NAC is a widely used pharmacological therapy in psychiatry in the treatment of neuropsychological disorders, including drug addiction (LaRowe et al., 2007; Mardikian et al., 2007; Gray et al., 2010; LaRowe et al., 2010), nicotine dependence (Knackstedt et al., 2009), pathological gambling (Grant et al., 2007), obsessive-compulsive disorder (Lafleur et al., 2006), schizophrenia (Berk et al., 2008b; Bulut et al., 2009), and bipolar disorder (Berk et al., 2008a) (for a review of NAC in psychiatric treatment (Dean et al., 2011)).
Antioxidant pretreatment with α-tocopherol and NAC attenuate the changes in anxiety-like behavior and oxidative stress markers caused by restraint stress in adult rats (Chakraborti et al., 2007). Anxiety-like behavior also associate with a differential increase in lipid peroxidation and decreases in GSH, SOD, and catalase levels in both male and female rats. Pretreatment with NAC has also been found to prevent learning and memory deficits (Lante et al., 2008) and ROS biomarkers (Lante et al., 2007) induced in the offspring of dams exposed to LPS during gestation. Pre-administration of NAC has also been found to attenuate the cerebral ischemia-reperfusion injury in a rat model of experimental stroke (Sekhon et al., 2003; Shen et al., 2003; Khan et al., 2004). Immunohistochemistry and real-time PCR studies demonstrate that NAC pretreatment reduces the gene expression of pro-inflammatory cytokines, such as tumor necrosis factor α (TNFα) (Feuerstein et al., 1994), interleukin 1β (IL-1β), and inducible nitric oxide synthase (iNOS) in LPS (lipopolysaccharides)-treated cells (Pahan et al., 1998). Postnatal administration of NAC has also been shown to reverse cognitive dysfunction and oxidative stress induced by bisphenol A (BPA), an environmental contaminant (Jain et al., 2011), and memory deficits in rats exposed to cadmium, an environmental heavy metal toxin (Goncalves et al., 2010).

Astaxanthin (AST), a naturally occurring carotenoid, is another antioxidant treatment that has received attention in the oxidative stress literature. AST has been shown to ameliorate aluminum chloride-induced oxidative stress and spatial memory impairments in mice (Al-Amin et al., 2016c). AST is also known to reduce oxidative stress and increase BDNF levels in the brain of aging mice (Wu et al., 2014) and rescue neuronal loss in rats (Lu et al., 2015).

Prenatal disruptions triggered by maternal administration of LPS during pregnancy was found to cause age- and region-specific redox dysregulation in the early postnatal brain
development in offspring (Al-Amin et al., 2016b). Prenatal LPS-exposed mice had elevated levels of lipid peroxidation, increased levels of advanced protein oxidative product, and higher levels of nitric oxide at P136 compared to healthy controls. They also showed depleted levels of antioxidant enzymes including SOD, catalase, and GPx (Al-Amin et al., 2016a). However, when adult offspring mice were given an antioxidant, astaxanthin, for a 6-week long treatment period from P91 to P132, antioxidant enzyme levels and oxidative stress markers were restored to normal levels in the brain and liver. Similar to ROS biomarkers, adult offspring behavior was found to be affected by LPS and alleviated by antioxidant treatment. Prenatal LPS-exposed mice displayed extensive immobility in the tail suspension test, indicative of depressive-like behavior in mice; frequent head-dipping in the hole-board test, a measure of anxiety-like behavior; and greater hypo-locomotion in the open field test. All of these behavioral outcomes were ameliorated by AST treatment (Al-Amin et al., 2016a).

Mechanisms linking prenatal stress and redox dysregulation

Maternal immune activation (MIA) and microglia

Potential mechanisms by which prenatal stress leads to redox dysregulation are explored in greater detail here. Stress experienced by a mother during pregnancy is a risk factor for neuropsychiatric disorders in offspring (King and Laplante, 2005; Khashan et al., 2008; Kinney et al., 2008; Bale, 2009) and MIA during pregnancy is also a risk factor for the same neuropsychiatric disorders (Brown et al., 2004; Meyer et al., 2006; Bilbo et al., 2018), suggesting that common maternal and offspring neurodevelopmental mechanisms may be involved. The mechanisms by which lasting, neurobiological alterations are induced in the developing fetal
brain by maternal physiological changes are not fully understood and are critically important for translational efforts to prevent psychiatric disorders.

Epidemiological studies suggest that exposure to prenatal stress increases the susceptibility of the offspring to schizophrenia and ADHD through MIA and alteration of the HPA axis (Udagawa and Hino, 2016). The link between maternal prenatal stress and risk of disease in offspring is thought to be related to MIA, wherein the mother’s immune system elevates maternal levels of cytokines, such as IL-8, IL-6, and TNF-α, but also peptides including corticotropin releasing hormone (CRH), glucocorticoids, and corticosterone (Weigent and Blalock, 1997; Brown et al., 2004). Pro-inflammatory cytokines, such as TNF-α, are known to generate large amounts of ROS (Shakibaei et al., 2005) and most ROS are membrane-permeable and able to cross the placenta to directly affect the embryo (Fantel et al., 1998). Whether cytokines cross from the maternal circulation through the placenta and into the fetal circulation (Zaretsky et al., 2004; Dahlgren et al., 2006; Ponzio et al., 2007) or trigger placental secretion of cytokines in the fetal compartment (Ratnayake et al., 2013), evidence suggests that the release of pro-inflammatory cytokines and stress-related hormones by the maternal immune system, hinder the normal brain development of the fetus (Nawa et al., 2000; Pearce et al., 2000; Marx et al., 2001).

MIA decreases the expression of a gene that regulates neuronal migration of GABAergic interneuron, Dlx, and increased oxidative stress gene expression in the fetus (Oskvig et al., 2012). As discussed previously, prenatal stress causes significant migration delays of GABAergic progenitor cells into the developing neocortex at E14 (Stevens et al., 2013). Exposure to the pro-inflammatory cytokine, IL-6, causes a similar GABAergic progenitor migration delay in non-stressed E14 mice, but the delay is not fully rescued by anti-IL-6
exposure concurrent with prenatal restraint stress (Gumusoglu et al., 2017a). This suggests that IL-6 has a significant, but non-essential role, in GABAergic progenitor migration delay in the embryonic brain. However, IL-6 is one of many pro-inflammatory factors implicated as a potential mediator of the effects of prenatal stress and MIA on offspring neurodevelopment.

Models of broad MIA have highlighted the importance of alterations in specific immune effectors, such as microglia, in fetal neurodevelopment. Microglia activation is regulated by both endogenous and exogenous factors (Kierdorf and Prinz, 2013) and they change their activation states to modulate their various cellular functions (for a review Subramaniam and Federoff (2017). The conversion from a steady-state, surveillance-like microglia phenotype to a pro-inflammatory, to an activation phenotype is regulated by two transcriptional factors: nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) and nuclear factor (erythroid-derived2)-like 2 (Nrf-2), both sensitive to redox dysregulation and signaling (Jazwa et al., 2011). Microglia are, therefore, both producers of ROS and modulated by oxidative stress. In other words, microglia themselves may affect the brain by producing ROS and through other redox mechanisms, so any change in microglia activation, such as those caused by prenatal stress, could also change redox conditions more broadly in the brain. Moreover, molecular and cellular processes that alter microglia, such as prenatal stress, could do so because of the redox molecules that also modulate microglia.

Microglia contribute to neurodevelopment and may be a significant component of effects of MIA and prenatal stress (Delpesch et al., 2015). In the adult mouse brain, microglia act similar to macrophages, traveling to the site of injuries and cleaning up cellular debris; however, their function during brain development is less clear. Microglial cell progenitors enter the central nervous system after the first fetal week and play a vital role in cortical development by
regulating the size of the neural progenitor cell pool (Cunningham et al., 2013). Prenatal stress and MIA both change microglia in neonatal rodent brain (Gomez-Gonzalez and Escobar, 2010; Van den Eynde et al., 2014), and MIA induces changes in their activation (Bilbo et al., 2018). Prenatal stress increases ramified microglia, a morphological phenotype of microglia characterized by two or more processes, in the neonatal brain but reduces amoeboid microglia, characterized by a round soma, in cortical regions (for a more detailed description of morphologies, please see Cunningham et al. (2013); Gumusoglu et al. (2017b)). In fetal brain, prenatal stress and interleukin-6 induce the most significant changes in the density of multivacuolated microglia—a type of microglia classified as having multiple vacuoles and/or pyknotic nuclei and a morphology relevant to early neurodevelopmental processes (Cunningham et al., 2013; Gumusoglu et al., 2017b). The maternal physiology underlying these microglial changes is not understood.

Because of the importance of this physiology (Bittle and Stevens, 2018), we set out to elucidate maternal factors that underlie the effects of prenatal stress on embryonic microglia in a mouse model. One maternal physiological component of prenatal stress is elevation of plasma corticosterone levels (Gomez-Gonzalez and Escobar, 2010). Known to readily pass the placenta (Beitins et al., 1973; Campbell and Murphy, 1977; Montano et al., 1993; Seckl et al., 2000), corticosterone is a glucocorticoid hormone elevated during MIA and the embryonic brain is known to express glucocorticoid receptors (Tsiarli et al., 2013). Previous work implicates corticosterone as a key mediator in effects of prenatal stress on the embryo (Field and Diego, 2008) and has been shown to influence microglia (Delpech et al., 2015). Prenatal stress also elevates pro-inflammatory cytokines, including IL-1β (Boksa, 2010), and the embryonic brain is known to be sensitive to maternal cytokines (Bauer et al., 2007; Gumusoglu et al., 2017b). MIA
upregulates multiple pro-inflammatory cytokines in the fetal brain, including IL-1β, IL-6, IL-17, IL-13, MCP-1, and MIP α, hours after MIA, but only IL-1β was shown to be elevated in the fetal brain 24 hours following the induction of MIA (Meyer et al., 2006; Garay et al., 2013). IL-1β plays a key role in regulation on microglia during their development (Meyer et al., 2006).

Glucocorticoids and IL-1β are important candidate mediators for prenatal stress effects on the embryonic brain.

To assess physiological candidates of a commonly used prenatal stress model that showed the greatest effect on microglia at embryonic day 14 (E14) (Gumusoglu et al., 2017b), we injected the stress hormone, corticosterone, and the pro-inflammatory cytokine, IL-1β, beginning on E12. We characterized microglia found within the developing cortical plate by morphology at E14, following 2 days of exposures (Diz-Chaves et al., 2012; Gumusoglu et al., 2017b). The timing of this exposure coincides with the first wave of invasion of the microglia into the neocortex (Swinnen et al., 2013). By E14, microglial cells are present throughout the brain parenchyma and are known to play a crucial role in the development of the cortex, including regulating the size of the neural progenitor cell pool (Cunningham et al., 2013). While not predicting that each factor could recapitulate stress effects in a reductionist way, we assessed whether they would have similarity to the effects of prenatal stress on microglia.

We found that corticosterone and IL-1β each had distinct influences on embryonic microglial cells compared to prenatal stress. We demonstrated that neither the exogenous IL-1β nor corticosterone recapitulated the full effects of prenatal stress on the morphology of microglial cells in the cortical plate of embryonic mice (for a full detailing of results, please see Bittle and Stevens (2018)). Briefly, we found that isolated physiological stress mediators, corticosterone, and IL-1β, induced increases in embryonic microglia in the cortical plate, but not
shifts in morphology as previously seen after prenatal stress. Interestingly, we found that two manipulations with overlapping antioxidant properties, sunflower seed oil and the antioxidant, N-acetylcysteine (NAC), moderated microglial changes with prenatal stress in the same way. The buffering of redox processes negated embryonic microglia changes, implicating oxidative stress among multiple stress-dependent mechanisms. These results are consistent with research that demonstrates that maternal oxidative stress status directly influences fetal microglia activation through gene expression changes and that maternal antioxidant status, in the case of maternal pretreatment with NAC, has protective effects on fetal microglia (Akhtar et al., 2017). These findings suggest that multiple physiological changes, including the dysregulation of endocrine, immune/inflammatory, and redox pathways, and their interaction, likely underlie prenatal stress as a risk factor for neurodevelopmental disruptions, including microglia (Diz-Chaves et al., 2012; Delpech et al., 2015).

As discussed previously, microglia are both producers of ROS and modulated by oxidative stress. In one study, microglia-activated inflammatory response in embryonic brain occurred 6-24 hour post maternal ethanol exposure in conjunction with GSH depletion and increased protein and lipid peroxidation (Akhtar et al., 2017). GSH depletion in cultured microglia also independently increased their oxidative stress and inflammatory responses. Therefore, it is hypothesized that microglia activation, inflammation, and redox dysregulation are interconnected mechanisms by which maternal exposure to environmental stressors are tied to fetal neurodevelopmental abnormalities. However, alterations to the developing brain after exposure to environmental stressors must have enduring consequences in order to explain the manifestation of neuropsychiatric illness during adulthood many years after the cessation of the stressor.
Glucocorticoids and cytokines

As discussed above, one maternal physiological element of prenatal stress is elevated plasma corticosterone levels (Gomez-Gonzalez and Escobar, 2010). Corticosterone has been shown to lead to redox dysregulation through the suppression of antioxidant enzymes in the brain (Zafir and Banu, 2009). Chronic administration of corticosterone showed a significant depression of antioxidant enzymes like SOD, catalase, glutathione-S-transferase, and glutathione reductase, in the brain of adult rats. Restraint-stressed rats also show a decrease in antioxidant enzymatic status, similar to that of the corticosterone injections. A significant increase in lipid peroxidation, a measure of oxidative damage to cell membranes, was observed in the corticosterone-treated animals and restraint stress animals (Zafir and Banu, 2009).

Prenatal stress also elevates pro-inflammatory cytokines and the embryonic brain is known to be sensitive to maternal cytokines (Bauer et al., 2007; Gumusoglu et al., 2017b). IL-6, a pro-inflammatory cytokine elevated during MIA, was found to have the ability to transfer from maternal circulation to fetal circulation via the human placenta in a bi-directional manner, but IL-1α and TNF-α showed nominal transfer through the placenta (Zaretsky et al., 2004). Radiolabeled IL-2 and IL-6 were shown to transfer from maternal circulation through the placenta to fetal circulation in rats (Dahlgren et al., 2006; Ponzio et al., 2007). While it remains unknown how readily other cytokines can cross the placenta, the secretion of cytokines from the placenta itself offers a second possible explanation for an elevation of cytokines in fetal circulation after MIA. In other words, MIA may trigger placental secretion of cytokines in the fetal compartment and alter fetal brain development indirectly (Ratnayake et al., 2013).
Pro-inflammatory cytokines, such as TNF-α, are known to generate large amounts of ROS (Shakibaei et al., 2005) in part by affecting mitochondrial membrane release which amplifies their signal and affect local signaling pathways. Under normal physiological conditions, these cytokine-induced ROS second messenger molecules would be degraded quickly by antioxidants to prevent unintended damage to other macromolecules. However, the embryo is highly sensitive to ROS injury because of its low antioxidant capacity (Wells and Winn, 1996; Wells et al., 1997; Wells et al., 2005; Shim and Kim, 2013). Therefore, in the absence of a sophisticated antioxidant defense system in the embryo, excessive ROS generation is left unchecked and can cause extensive and indiscriminate damage to surrounding cells and tissues.

Maternal prenatal stress induces MIA (for a review Howerton and Bale (2012)) which, in turn, activates microglia and generates pro-inflammatory cytokines and glucocorticoids. I hypothesize that the embryo is particularly vulnerable to MIA-induced ROS and unable to effectively neutralize the onslaught of pro-inflammatory cytokines and glucocorticoids with its underdeveloped antioxidant defenses. The objective of this thesis is to understand the complex relationship between the embryonic cellular and molecular mechanisms involved in inhibitory neuron development that are modified by prenatal stress, the role of redox regulation in the embryonic brain as the result of the prenatal stress, and the long term effects of redox components of prenatal stress on behavior and the development of forebrain GABAergic systems in the adult.
Summary and hypotheses

In summary, I have reviewed how prenatal stress, through alterations in GABAergic system development, may increase the risk of offspring developing neuropsychiatric disease. I have also outlined studies that show that migrating cortical interneuron progenitor cells are sensitive to OXPHOS disturbances. OXPHOS disturbance may in fact influence redox balance and which may play a role in the interneuron migration delay observed after prenatal stress. Mitochondrial disruptions in OXPHOS lead to imbalances in cellular redox states and these disturbances have been shown to influence development via fetal programming mechanisms and thereby increase the risk of adult neuropsychiatric disease. I hypothesize that a shift in redox balance mediates the effects of prenatal stress on the GABAergic neural development. There is an increasing appreciation for the role inhibitory neural circuitry plays in the development of neuropsychiatric illness. Prenatal stress leads to alterations in these systems and I hypothesize that the relationship between prenatal stress and alterations in postnatal cortical GABAergic populations is mediated by oxidative changes in the embryonic brain during development. Redox dysregulation is related to neuropsychiatric illnesses such as schizophrenia (Do et al., 2009) and ASD (Walker et al., 2015); diminished expression of PV+ GABAergic neurons in the adult cortex (Powell et al., 2011); smaller neural progenitor pools in the embryonic cortex (Nayernia et al., 2017); modified induction of transcription of genes related to oxygen sensing, cell differentiation, cell proliferation (Yoneyama et al., 2010); and altered mRNA levels of neuronal and neural progenitor markers in neurogenic regions of the cortex (Nayernia et al., 2017). Redox dysregulation is also known to affect histone functioning and modifies DNA methylation, which, in turn, can lead to suppression of mRNA transcription and protein
synthesis. I also hypothesize that the impacts of prenatal stress on adult behavior are mediated by oxidative change in the embryonic brain during development which may be a common mechanism of fetal programming.

**Significance and aims**

Maternal stress during pregnancy is associated with increased risk of psychiatric disorders in offspring (King and Laplante, 2005; Bale, 2009; Kim et al., 2015), but embryonic brain mechanisms disrupted by prenatal stress are not fully understood. GABAergic system functioning has been implicated in a number of psychiatric disorders such as schizophrenia and autism (Benes and Berretta, 2001; Hashimoto et al., 2008; Yip et al., 2008) and research shows that disruption of inhibitory neural circuitry during fetal development can have crucial, long-lasting consequences for neuronal function in adolescence and adulthood. Thus, alterations to the developing brain during prenatal stress must have long-lasting effects in order to explain the emergence of neuropsychiatric illness during adolescence and adulthood, many years later. Therefore, there is a critical need to understand the mechanisms by which GABAergic systems are altered by prenatal stress.

Redox dysregulation has been shown to be a mediator of the impact of multiple environmental exposures on biological systems, making it a prime candidate for mediating the effects of in utero environmental impacts on the embryonic brain like prenatal stress (Wells et al., 2009). The balance between oxidative and reductive processes is critical during rapid periods of cell growth and differentiation, so fluxes in the equilibrium of reactive oxygen species (ROS) during embryonic brain development could have significant effects (Thompson and Al-Hasan, 2012). The concept of pro-oxidant-antioxidant homeostasis is critical to the understanding of the
deleterious effects of ROS overproduction. The notion of a disruption, or a dysregulation, of the redox balance suggests that problems could arise with a perturbation on either side of the equilibrium (unusually high generation of ROS or deficiencies in the endogenous antioxidant defenses). Consequently, a complex system of endogenous antioxidant defenses has evolved that corrects this redox imbalance and restores the pro-oxidant-antioxidant homeostasis (Burton and Jauniaux, 2011). Since I hypothesize that prenatal stress tips the balance in favor of the pro-oxidant state which subsequently impacts GABAergic progenitor migration, I aim to develop methods by which I could change this redox imbalance, both with direct manipulations of pro-oxidants and with maternal treatments of antioxidants during the prenatal restraint stress. With these methods, I predict that redox manipulations will change embryonic brain ROS and GABAergic interneuron migration, demonstrating the sensitivity of cortical interneuron migration to redox balance.

The overall goal of this project is to investigate the role that redox dysregulation plays in the effects of prenatal stress on the developing brain. The purpose of Aim 1 is to examine the effects of redox dysregulation on the migration of GABAergic progenitor cells in the embryonic brain with one important method to test whether maternal treatments with antioxidants before prenatal stress ameliorate the deleterious effects caused by prenatal stress in the embryonic brain. I hypothesize that redox dysregulation mediates the effect of prenatal stress on the migration patterns of interneuron progenitors. Therefore, I predict that maternal antioxidant treatment will rescue alterations in cortical interneuron migration due to prenatal stress. The purpose of Aim 2 is to investigate the effects of prenatal stress on behavior and postnatal GABAergic neuron populations in adult offspring brain. I hypothesize that redox dysregulation mediates the effect of prenatal stress on anxiety-like behavior, social behavior, sensorimotor behaviors, and
GABAergic cell populations. Therefore, I predict that maternal treatments of antioxidants during prenatal stress will ameliorate aberrant behaviors observed due to prenatal stress effects and restore GABAergic cell populations.

This thesis is innovative because it seeks to elucidate one of the mechanisms during embryonic development by which prenatal stress affects the embryonic brain as evidenced by the development of inhibitory neural systems and postnatal behaviors. Several studies have shown the prenatal stress has a causal role in the neural circuitry in the mature brain and adult psychopathology (Huot et al., 2004; King and Laplante, 2005; Weinstock, 2008; Bale, 2009; Harris and Seckl, 2011). Numerous studies have also shown that redox dysregulation contributes to neurological disorders (Peuchant et al., 2004; Cambonie et al., 2007a; Derks et al., 2010b; Ziech et al., 2011). This project is the first to investigate whether disturbances in the redox state during prenatal development act as an intermediary between the effects of prenatal stress and altered GABAergic development in the embryonic and adult brain. This project will address these questions and fill the gap in our knowledge regarding the cellular and molecular mechanisms that are modified by prenatal stress. The ultimate goal is to advance our knowledge of the risk factors for mental illness, including genetic, biological, and environmental causes and, equipped with this knowledge, develop better treatments and interventions for those at risk for psychiatric disorders.

Aim 1: Determine whether redox dysregulation is involved in the effects of prenatal stress on the migration of GABAergic interneuron progenitors into the embryonic brain

Many studies have demonstrated that prenatal stress has a variety of deleterious effects on the development of inhibitory neuron systems in the embryonic and neonatal brain (for a
review see (Fine et al., 2014)). Inhibitory neuron progenitors migrate a great distance from their subcortical birthplace in the medial and caudal ganglionic eminences (MGE and CGE) before taking up residency in the cerebral cortex (Polleux, 2002). Prenatal stress is known to decrease transcription factors for GABAergic migration (Stevens et al., 2013) and decrease gene expression of cortical brain derived neurotropic factor, BDNF—a growth factor that acts as a permissive cue for interneuron migration into the cortex and influences GABAergic maturation in forebrain—by 53% ($p = 0.043$, unpublished pilot data). Research also suggests that prenatal stress is associated with a lower density of GABAergic progenitors in the cortical plate (Stevens et al., 2013) and that extracellular factors like BDNF affect tangential migration (Polleux, 2002).

The tangential migration from MGE/CGE to the cerebral cortex primordium requires a great deal of energy from mitochondria via ATP production generated through OXPHOS. Migrating interneurons are especially sensitive to disruptions in OXPHOS in ways glutamatergic projection progenitor cells are not. It is believed that the greater distance tangentially migrating GABAergic progenitor cells must travel from their subcortical birthplace is what makes them more vulnerable to disruptions in OXPHOS than the radially migrating glutamatergic progenitor cells (Lin-Hendel et al., 2016). Highly reactive forms of oxygen are produced by the body during normal metabolic reactions and processes (Guerin et al., 2003) and their high metabolic demands lead to the creation of free radicals and the leakage of electrons from the electron transport chain onto molecular oxygen creating ROS (Burton and Jauniaux, 2011; Lin-Hendel et al., 2016). This disturbance in the equilibrium of pro-oxidants and antioxidants may be responsible for findings that mitochondrial dysfunction in inhibitory neuron precursors causes a delay of migration into the cortex and may through this and other mechanisms delay inhibitory precursor migration after prenatal stress.
Pilot data obtained from microarray analysis of the embryonic day 13 (E13) ganglionic eminence (the origins of cortical interneurons) revealed that prenatal stress significantly changed the expression of 5,000 genes. The expression of a significant number of Complex 1 genes in mitochondria was changed (unpublished pilot data), suggesting changes in cellular energy generation and the by-production of ROS. Pathway analysis of all 5000 genes using PANTHER identified the “oxidative stress response” pathway as one of the top five pathways disproportionately altered due to prenatal stress and found that four genes involved in the “antioxidant pathway”—catalase, glutathione peroxidase, sestrin 1 and sestrin 3—were significantly changed (unpublished pilot data).

Endogenous antioxidants generated by the body and exogenous nutritional antioxidants supplied in the diet are known to combat ROS. Maternal nutritional factors during pregnancy are linked to fetal brain development and later offspring behavior (Zheng et al., 2015). Maternal dietary supplementation of antioxidants, like hydroxytyrosol, during pregnancy has been shown to have protective effects against redox dysregulation and mitochondrial dysfunction in prenatally-stressed adult rat offspring (Feng et al., 2012; Zheng et al., 2015). For the purpose of these experiments, two antioxidant treatments were chosen, N-acetylcysteine (NAC) and astaxanthin (AST), because of their broad effects on redox dysregulation and ability to combat the toxic effects of redox dysregulation induced by prenatal infections, teratogens and inflammation (Dennery, 2007; Wells et al., 2009). NAC is a common exogenous antioxidant that contributes to making the important metabolic antioxidant, glutathione (Olsson et al., 1988) and has been shown to scavenge pro-oxidants directly (Aruoma et al., 1989). It has been used in a number of studies as pharmacological treatment of psychiatric conditions that result from redox dysregulation (for a review, please see (Dean et al., 2011)). AST, a xanthophyll carotenoid, is a
potent antioxidant that is known to cross the blood brain barrier and scavenge ROS to improve brain function. It possesses neuroprotective properties by restoring the antioxidant enzymes SOD and GPx and reducing lipid peroxidation (Liu and Osawa, 2009; Wu et al., 2014; Al-Amin et al., 2016a).

It has been demonstrated that NAC and AST rescue behavioral deficits induced by maternal LPS exposure, such as hypolocomotion in the open field task, depressive-like behaviors on the tail suspension task, and anxiety-like behavior on the hole-board task, in adult offspring mice whose mothers were administered treatment during their pregnancies and also rescue other neurobiological deficits caused by ROS during development (Palozza and Krinsky, 1992; Odabasoglu et al., 2008; Wells et al., 2009; Dean et al., 2011; Zheng et al., 2014; Al-Amin et al., 2016a). However, it has yet to be elucidate whether NAC and AST can rescue the behavior deficits associated with prenatal stress.

**Aim 1.1: Determine whether pro-oxidants induce aberrant behavior of migrating GABAergic interneuron progenitors.** The purpose of Aim 1.1 is to use an *in vitro* method to measure GABAergic interneuron progenitor cell migration in embryonic forebrain and examine the effects of hydrogen peroxide (H$_2$O$_2$), a potentially harmful ROS, on the migration movements of these cells into the cortical plate. I hypothesize elevations in ROS will slow the migration cortical interneurons. This sub-Aim will allow me to test directly whether pro-oxidants can induce directional and velocity changes in migrating GABAergic interneuron progenitors.

**Aim 1.2: Examine in utero manipulations of pro-oxidant states in the embryonic brain.** Aim 1.2 serves to evaluate the effects of an *in vivo* manipulation to create a pro-oxidant environment in the embryonic brain by administering rotenone, a known inhibitor of Complex I
in the mitochondria and generator of ROS, directly into the in utero embryonic brain. This sub-Aim will allow me to test the hypothesis that rotenone will cause elevated levels of pro-oxidants in embryonic forebrain tissue and will lead to delays in the tangential migration of GABAergic interneuron progenitors into the cortical plate.

**Aim 1.3: Measure ROS, antioxidant activity, redox state, and gene expression of endogenous pro- and antioxidants after prenatal stress and determine whether maternal antioxidants alter these measures in the embryonic brain.** In order to address the hypothesis that prenatal stress affects neural migration by dysregulating redox balance in the embryonic brain, I will directly measure pro-oxidant and antioxidant activity in embryonic brain by means of enzyme activity and gene expression assays. One of the important contributions of this sub-Aim is the validation of maternal antioxidant administration as a means of changing embryonic brain redox state.

**Aim 1.4: Determine whether maternal antioxidant treatment during prenatal stress alters the migration patterns of GABAergic interneuron progenitors in the embryonic neocortex primordium.** Aim 1.4 serves to measure tangential migration of GABAergic interneuron progenitors into the developing neocortex of embryos and assess the effects of maternal antioxidant treatments during prenatal stress on the tangential migration. I hypothesize that migration of GABAergic progenitor cells will be delayed as the result of prenatal stress because it dysregulates redox balance, leading to the prediction that maternal antioxidant treatment during prenatal stress will ameliorate this delay.

The overall purpose of Aim 1 is to examine the effects of redox dysregulation and prenatal stress on the migration of GABAergic progenitor cells in the embryonic brain and determine whether redox balance during prenatal stress plays a central role in effects on
GABAergic progenitor migration. I will use an array of methodology and approaches, including maternal antioxidant administration, to test the hypotheses laid out in this chapter.

**Aim 2: Determine if redox dysregulation is involved in the effects of prenatal stress on behavior and postnatal GABAergic neuron population outcomes in adult offspring brain**

Prenatal stress is known to cause a range of inhibitory or anxiety-like behaviors in rodent offspring which include reduced social recognition (Harmon et al., 2009), increased anxiety-like behavior in the light-dark box and the elevated plus maze (Laloux et al., 2012; Grigoryan and Segal, 2013), and reduced activity in the center of the open field (Laloux et al., 2012; Grigoryan and Segal, 2013). Previous research findings published by our lab suggests that prenatal stress also alters adult CD1 mouse offspring behavioral outcomes and that the altered behavior is associated with neurobiological differences in individual mice. Parvalbumin+ (PV+) cells are a specific subtype of GABAergic interneuron that denote maturation into a critical component of cortical function and are important to our investigation of the appropriate differentiation of mature cortical GABAergic populations. Our lab found that prenatal stress decreased the ratio of PV+/GAD67 cells in hippocampal CA, but increased the ratio in medial frontal cortex (mFC) in adult mice (Lussier and Stevens, 2016). We also found that prenatal stress decreased the *Pv/Gad1* gene expression ratio in adult brains, but only in medial frontal cortex (Lussier and Stevens, 2016). In assessing behavior, our lab found that prenatally-stressed mice spent more time in the closed arm of the elevated plus maze and less time in the center of the open field on Day 2 (Lussier and Stevens, 2016). Additionally, there was a significant positive correlation between multiple anxiety-like behavioral abnormalities with the total GABAergic cell density and the ratio of PV+/GAD67GFP+ cells in mFC and hippocampus for adult offspring. These
findings suggest that prenatal stress affects mature GABAergic cell populations which may be linked to prenatal stress impacts on adult anxiety-like behavior.

Redox dysregulation during prenatal stress may be responsible for the effects on adult development in behavior and in mature GABAergic populations. The idea that antioxidants can mitigate the effects of in utero stress exposure on adult anxiety-like behavior is not a novel one. Research shows that AST has neuroprotective properties against the behavioral deficits seen in prenatal maternal LPS exposure in mice (Al-Amin et al., 2016a) and prenatal administration of NAC and AST have been shown to affect adult offspring behavior (Lante et al., 2008). Furthermore, they reduce ROS markers in brains of adult offspring mice (Al-Amin et al., 2016a), making them good candidates for maternal prenatal intervention treatment.

**Aim 2.1: Evaluate the behavioral outcomes secondary to prenatal stress and the impact of maternal antioxidant treatment during prenatal stress on anxiety-like behaviors, social behaviors, and behaviors in the sensory-motor domain in adult offspring mice.** Aims 2.1 seeks to evaluate behavioral outcomes of adult offspring mice and the impact of maternal antioxidant treatments during prenatal stress on these behaviors. This sub-aim serves to test adult offspring mice for: 1) an anxiety-like phenotype using the open-field test and the elevated plus maze, 2) social approach in the three-chamber sociability and social preference task, and 3) behaviors in the sensory-motor domain using the open field test, rotarod performance test, water T-maze task, and pre-pulse inhibition test. I hypothesize that prenatally-stressed offspring male and female mice will show a more anxious-like phenotype, display less sociability, and perform more poorly on the sensorimotor tasks than the non-stressed offspring mice. I hypothesize that maternal NAC and AST antioxidant administration will rescue these behavioral deficits and restore behavior to levels of the non-stressed control mice.
Aim 2.2: Assessment of total GABAergic cell populations and the PV subtype in hippocampal and mFC regions of prenatally-stressed adult offspring mice and evaluate the impact of maternal antioxidant treatment during prenatal stress. The purpose of Aim 2.2 is to use stereological assessments of hippocampal and mFC densities of GAD67-GFP+ cells and PV+ cells. This Aim seeks to determine whether maternal administration of antioxidants during prenatal stress can change adult offspring behavior, which would suggest alterations to the embryonic brain that have long-lasting functional effects. I hypothesize that the mFC and hippocampal PV+ and GAD67+ neuron populations will be altered in the adult brain, and maternal antioxidant treatments during prenatal stress will abrogate redox dysregulation in the embryonic brain, and prevent changes in cell densities in the adult brain.

Aim 2 has the potential to shed light on whether redox dysregulation during pregnancy mediates the effect prenatal stress has on anxiety-like behaviors, social behaviors, sensorimotor behaviors, and postnatal GABAergic neuron populations in the brains of adult offspring mice. Maternal treatments of antioxidants during prenatal stress may have long-lasting effects on the embryonic brain and those effects may manifest alterations in adult offspring behavior and may be evident in postnatal GABAergic PV+-and total GAD67+ neuron populations.
CHAPTER 2. METHODS

Mice

GAD67-GFP+/- knock-in mice were bred on a CD1 background and housed in accordance with the University of Iowa 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.

Treatments and prenatal stress

On E12, some prenatally-stressed and non-stressed dams were injected intraperitoneally (ip) with either: 1) N-acetylcysteine (NAC) (200 mg/kg of 40 mg/ml saline solution with 30% sodium hydroxide [1 μM] to bring pH to 7.4; Sigma Aldrich, St. Louis, MO A7250-25G) (Ho et al., 1999; Bielefeld et al., 2007; Davis et al., 2010; Flurkey et al., 2010; Azuelos et al., 2015), 2) astaxanthin (AST; 30 mg/kg [1st daily injection] and 10 mg/kg [2nd and 3rd daily injections] in a 10 mg/ml saline solution; with 3% dimethyl sulfoxide [DMSO]; Sigma Aldrich, St. Louis, MO SML0982) (Suzuki et al., 2006; Lee and Lee, 2011), or 3) saline (200 μl, 0.9% saline), 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 three to four hour intervals, starting on E12 and continuing until tissue collection (Stevens et al., 2013; Lussier and Stevens, 2016). On E13 or E14, pregnant
dams underwent one additional restraint session prior to embryo collection. All pregnant female mice were euthanatized by ketamine/xylazine anesthesia followed by rapid decapitation.

It is important to note here that consideration of half-life and absorbance rates was made in the maternal dosing of NAC and AST. Due to the 20 hour half-life of AST (Hashimoto et al., 2009), the restraint stress protocol of stressing three times a day, and injections 20 minutes prior to restraint stress, pregnant dams were given an initial injection of 30 mg/kg and then 10 mg/kg for the 2nd and 3rd injections so as to both allow for peak levels of AST at the time of stress and avoid any toxic effects of excess AST. NAC, on the other hand, has a shorter half-life of approximately 11 minutes in rodents (Dickey et al., 2008). As such, all three injections of NAC were of the same concentration to provide for a peak level at the time of stress.

**Live imaging**

At E14, GAD67GFP+ cells were visualized in live brain slices as they migrated tangentially from the medial ganglionic eminence (MGE) towards the developing neocortex. Embryonic mouse brains from non-stressed dams were dissected and sectioned with a vibrating microtome (350-450 μm thick) in cold artificial cerebral spinal fluid (aCSF) to create at least two coronal sections of forebrain. Embryos from five independent litters were used to measure migration of GABAergic interneuron progenitors, using matching sections from multiple embryos of each litter in control and experimental media: (1) standard aCSF as the control media (5% fetal bovine serum in Hanks’ solution [Life Technologies, USA, catalog # 14175079], supplemented with D-glucose [6 mg/ml; Sigma-Aldrich, St. Louis, MO, G7021-100G] and streptomycin [20 mg/l; Sigma-Aldrich, St. Louis, MO, S9137-25G]) and (2) aCSF containing the pro-oxidant, hydrogen peroxide (1 μM or 5 μM concentrations; Sigma-Aldrich, MO, H1009-
100ml) (Feeney et al., 2008). Tissues were mounted between a glass coverslip and flexible mesh within a plastic chamber and left at room temperature for one hour. Time-lapse imaging was then performed of migrating interneurons using a Leica SP5 scanning laser confocal microscope equipped with automated image capture (Dailey et al., 2013) and a stage with a hot air supply to maintain a temperature of 36-38°C. Z-stacks (3 μm z-step interval) were taken at 20x magnification and across 45 μm every 5-10 minutes over 1-2 hours focused on the lateral, anti-hem region of the cortical plate. Multiple tissue sections were imaged in parallel to maximize data collection. Images were analyzed using ImageJ to assess the direction and rate of movement of individual GABAergic progenitor cells in the superficial and deep migratory streams. Results were based on the average measurements of 10 migrating cells in five coronal tissue slices in each control and experimental group.

**Embryonic intracerebroventricular (ICV) injection**

Pregnant dams with E13 embryos were anesthetized with 3% isoflurane vaporizer and a midline incision, approximately 3 cm in length, was made along the abdomen. The uterus was extracted from the abdominal cavity with moisturing DPBS. Either rotenone (0.031 μg/μl in 0.9% NaCl, Enzo Life Sciences, ALX350360G001), an inhibitor of mitochondrial respiratory chain complex I (Palmer et al., 1968), or 0.9% NaCl was injected into one of the lateral ventricles of the embryonic forebrain using a glass micropipette. Evan’s Blue dye (1%, MP Biomedicals, catalog # 0215110805) was included in the injection to ensure visualization successful delivery into the embryonic lateral ventricle. A total of 1 μl of rotenone solution or saline vehicle was injected into each embryo. After ICV injection of all embryos, the uterus was returned to the abdominal cavity and the abdominal wall and skin were closed with sutures. On
E14, 24 hours after ICV injections, pregnant dams were euthanized and whole embryonic heads were collected for measurement of GABAergic progenitor migration as described below.

**Gene expression**

To examine the source of migrating GABAergic progenitors, ventral forebrain tissue was dissected from E13 embryos and flash frozen on dry ice. Quantitative PCR was performed to assess changes in the expression of redox related genes, as well as genes involved in interneuron development: thioredoxin reductase 1 (Tr1; F: 5’-GACCAGGAAACCAAGGGAG-3’ R: 5’-CACGCGTGTCATCAACATC-3’), glutathione peroxidase 1 (Gpx1; F: 5’-AGTGCAGTGAATGGTGAAGA-3’ R: 5’-GCACACCGGAGACCAAATGA-3’), sestrin 1 (Ses1; F: 5’-GGCCAGGACGAGAAGAATGA-3’ R: 5’-AAGGAGTCTGCAAATAACGCAT-3’), sestrin 2 (Ses2; F: 5’-GGATTATATACCTGGGAAGACC-3’ R: 5’-CGCAGTGATGTAGTCC-3’), sestrin 3 (Ses3; F: 5’-CCAGGACTACACCTGGGAAA-3’ R: 5’-AACCTTCAGGCTCCGTTCA-3’), catalase (Cat; F: 5’-CGCAATTCACACCTACACGC-3’ R: 5’-TTTCCCTTCAGGAAACGGCA-3’), superoxide dismutase 1 (Sod1; F: 5’-CAGGACCTCATTTTAATCTTAC-3’ R: 5’-CCCAGGTCTCCAACTGC-3’), nuclear factor erythroid-derived 2 (Nrf2; F: 5’-CCACATTTCCCTTCTGTTTG-3’ R: 5’-GACACTTCCAGGCGGCACTATCT-3’), Apoptosis-inducing factor 1, mitochondrial (Aifm1; F: 5’-CGCTAAGCCATACTGGCATCA-3’ R: 5’-CAACTGTGGGCAAAACTATCCA-3’), isocitrate dehydrogenase [NADP], mitochondrial (Idh2; F: 5’-TGCCGTGCTTGATCCATGG-3’ R: 5’-GGAAGTGCTCGTCAGCTTCC-3’), NADH dehydrogenase [ubiquinone] iron-sulfur protein 4, mitochondrial (Ndufs4; F: 5’-GAGCACATCCCTGGGAAGC-3’ R: 5’-
GATGTGCTCTTCTGGAACACC-3’), and C-X-C chemokine receptor type 4 (Cxcr4; F: 5’-ATGGAACCAGTGTGAGT-3’ R: 5’-TGAAGTAGATGCTGGAACACC-3’). RNeasy Mini Kit (Qiagen, Valencia, CA, catalog # 74134) was used per the manufacturer’s protocol to extract mRNA from the tissue. Total RNA concentrations were quantified on a spectrophotometer (Nanodrop, Thermo Scientific, USA) and reverse transcribed using Transcriptor First-Stand cDNA Synthesis Kit (Roche, USA, product # 04897030001). qPCR with Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, Warrington, UK, catalog # 4367659) was performed using Applied Biosystems Model 7900HT instrument. The endogenous gene, glyceraldehyde-3-phosphate dehydrogenase (Gapdh; F: 5’-GGTGAAGGTGCGTGTTGAAAG-3’ R: 5’-CTCGCTCCTGGAAGATGGTG-3’), was used as a control gene and relative expression was calculated as: \(2^{-|Ct_{[\text{gene of interest}]} - Ct_{\text{GAPDH}}|}\), where Ct is threshold cycle.

**Glutathione assay**

The ratio of reduced glutathione (GSH) to glutathione disulfide (GSSG) is a common indicator of redox status and a gauge of antioxidant efficiency. Total GSH and GSGG content was determined as previously described (Tietze, 1969; Griffith, 1980), respectively, using spectrophotometry. Total forebrain tissue from E13 embryos was pooled (2-3 forebrains per sample) and homogenized in 5-Sulfosalicylic acid. The BCA Protein Assay Kit (Thermo Scientific, Rockford, IL, catalog # 23225) was used to determine protein content and for normalization of samples.
Antioxidant activity

The following antioxidant enzyme activity assays were performed on E13 total forebrain. Upon collection, forebrains were homogenized in 50 mM potassium phosphate buffer, pH 7.8, with 1.34 mM diethylenetriaminepentaacetic acid (DETAPAC). Glutathione peroxidase 1 (GPx1), an antioxidant enzyme that enzymatically reduces hydrogen peroxide and organic hydroperoxides to water to limit its harmful effects (Carter et al., 2004), was measured spectrophotometrically. Enzymatic activity of GPx1 was determined by using 2.5 mM H$_2$O$_2$ as substrate and absorbance was read at 340 nm continuously for 2.5 minutes (Lawrence and Burk, 1976). One unit of activity was defined as the amount of protein that oxidizes 1 μM of NADPH per minute. Protein concentrations were determined by the Lowry assay.

An essential enzyme in the oxidoreductase system, thioredoxin reductase (TR), helps combat ROS and was detected spectrophotometrically with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) as substrate using 5 mM DTNB and 0.2 mM NADPH in 0.1 M potassium phosphate, 1 mM EDTA, pH 7.0 plus 0.1 mg/ml bovine serum albumin. Absorbance was read at 412 nm for 6 seconds (as previously described by (Holmgren and Bjornstedt, 1995; Karimpour et al., 2002)).

Dihydroethidium (DHE)

Embryonic brain tissue was examined for dihydroethidium (DHE) fluorescence, an indicator of superoxide (Kimura et al., 2010). When DHE comes into contact with the superoxide anion in tissue, the cells fluoresce. Antimycin-A, a known inhibitor of Complex III of the mitochondrial electron transport chain, was used as a positive control because of its ability to increase one electron reduction of O$_2$ to form superoxide. E13 embryo head tissue was embedded
fresh in Optimal Cutting Temperature (OCT) compound and coronally cryosectioned (Leica, 1510-3, Bannockburn, Illinois) at 10 μm. Sectioned tissue was immediately frozen on slides. As a positive control, 10 μM antimycin-A (Sigma Aldrich, St. Louis, MO, A8674) and 10 μM DHE (Life Technologies, Carlsbad, CA, D1168) in 0.1% DMSO was applied to one tissue sample and incubated at 37º C, 5% CO₂ for 10 minutes (Coleman et al., 2014). After 10 minutes, slides were gently rinsed with PBS (containing 5 mM pyruvate), coverslipped, and immediately imaged with an epifluorescent microscope (Zeiss) with a filter cube for red detection (587 excitation/649 detection) with digital camera and Stereoinvestigator software for digital imaging (Microbrightfield, Vermont). 10 μM DHE was applied to a second sample, the tissue incubated for 10 minutes, and then rinsed with PBS before coverslipping and imaging. Gain intensity and exposure time settings for digital imaging were established using control tissue with antimycin-A as a maximum intensity measurement and maintained while imaging control embryonic brain (with and without antimycin-A) immediately followed by embryonic brain from a comparison experimental group. Images of DHE+ cells were obtained using the 40x objective from three different places in the ganglionic eminence and were analyzed for fluorescence with ImageJ software. Within each brain, mean fluorescent intensity (MFI) levels from a total of 300 cells across multiple scattered regions within the ganglionic eminence, the origin of GABAergic progenitors, were averaged. The average level from each experimental condition sample was normalized to average level in the matched non-stress control processed at the same time and compared across groups.
GABAergic progenitor migration

Pregnant dams were sacrificed and whole embryonic heads were collected and fixed in 4% paraformaldehyde (PFA) overnight and incubated in 20% sucrose for at least 20 hours. Tissue was embedded in OCT, cryosectioned at 25 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 one hour. They were then incubated overnight with 5% goat serum/PBS++ and primary antibody, anti-GFP (1:1000, Abcam, AB13970). 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 (4′,6-diamidino-2-phenylindole, Vector Laboratories, #H-1200). Migration of GAD67GFP+ cells in coronal tissue sections was measured using fluorescent microscopy with a Zeiss AxioImager M2 microscope with Stereoinvestigator software. We measured the circumferential length of the superficial stream of GAD67GFP+ cells along the edge of the dorsal forebrain as a percentage of the entire circumferential length of the cortical plate and averaged across at least four anterior to posterior sections. Average percent migration were compared between antioxidant, stress, pro-oxidant, and control groups.

Treatments and prenatal stress for adult offspring mice

Beginning on E12 and for the duration of their pregnancies, some prenatally and non-stressed dams were given ip injections of either: 1) NAC, 2) AST, or 3) saline, 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 three to four hour intervals (Lussier and Stevens, 2016). All pregnant dams 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 6-8 pups with even distribution of male and female pups. On P24, mice were weaned from their mothers and then single-sex group-housed.

**Behavioral tests of adult offspring mice**

All behavioral tests were performed on 10-14 week old male and female offspring mice. During the light cycle, mice were allowed to habituate to the testing room 30 minutes prior to the testing. All behavioral tests were conducted on consecutive days with only one test per day. 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. Testing apparatus was cleaned with 70% ethanol and dried prior to each daily assessment and between the assessment of each mouse.

**Open field test (OFT)**

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). 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, 14-inch long closed arms with 8-inch tall walls and two open arms without any walls for five minutes on a single day. The amount of time spent in and 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 (Fig. 1). During the first phase (Fig. 1A), the doors to the other chambers were closed and, for 10 minutes, the experimental mouse was allowed to habituate to the center chamber.

Figure 1. Social approach phases
During the second phase or the “sociability” phase (Fig. 1B), 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 and Anymaze software video-recorded the movements of the experimental mouse as the mouse traversed all three chambers for 10 minutes. The amount of time spent in and entries into close proximity to the “mouse” and the wire cup in the other chamber (“empty cup”) were recorded and analyzed statistically.

During the third phase or the “social recognition” phase (Fig. 1C), 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. For 10 minutes, the experimental mouse was allowed to traverse all three chambers and the movements of the experiment mouse were video-recorded by Anymaze software. The amount of time spent in and entries into close proximity to the “familiar mouse” and the “novel mouse” were recorded and statistically analyzed. Social approach task was performed to examine general sociability and recognition of social novelty. Normal, wild-type mice prefer to spend more time with another mouse (sociability) compared to an inanimate object and will show interest in a novel, stranger mouse more so than a familiar mouse (social novelty/social recognition) (Lan et al., 2011; Faizi et al., 2012).

**Rotarod**

Over the course of three days, offspring mice were tested on the rotarod apparatus (Stoelting) for five trials every day, each separated by 20-30 min breaks. The mice were placed on a horizontally-oriented, rotating cylinder (rod) suspended above a platform and the speed of
the rotarod was mechanically driven and accelerated over time for four minutes. Mice began the task at 4 rotations per minute (rpm) and accelerated to 80 rpms over four minutes. The length of time the mice stayed on the rotating rod was measured with a maximum time of five minutes if mice persisted. The rotarod task was used to assess motor behavior and procedural learning in the offspring mice.

**Water T-maze**

Offspring mice were placed between the walls of a plastic T-maze within a water tank heated to 22 degrees Celsius infused with white paint to obfuscate the platform hidden beneath the water’s surface.

During the Training Phase, mice were trained to swim to find a hidden platform in an assigned arm of the maze in a room with distal visual cues (Fig. 2). Once mice reached the hidden platform on five consecutive trials with no wrong-arm entering errors, mice reached “criterion.” Mice completed four additional days of training after reaching criterion.

The second phase of the task, known as the Reversal Phase (Fig. 2), occurred approximately 24 hours after the mice completed the final training Trial. This phase consisted of the hidden platform being moved to the opposite arm of the T-maze and the number of wrong turns and the seconds to reach the platform were measured over the course of 10 trials on a single
day. The Reversal Phase was performed to evaluate the perseverance and cognitive flexibility in learning in the offspring mice.

**Prepulse Inhibition**

Over the course of a single day, mice were subjected to prepulse inhibition task. Mice were placed 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 proceed with 90 different trials broken up over 3 blocks. The first block consisted of five 20msec 120 dB pulse alone (no prepulse) trials. The second block consisted of ten 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 GAD67GFP+/- offspring adult offspring mice from behavioral testing (Aim 2.1) were used for immunohistochemistry. Four weeks after the final testing day, to avoid effects of testing stress, brains were collected for examination. Adult offspring mice were perfused first with cold PBS and then with 4% PFA. Brains were collected from male and female mice and placed directly into 4% PFA. After overnight PFA incubation, brains were rinsed in
PBS and then transferred to 20% sucrose for at least 20 hours. Brains were embedded in OCT, 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 one hour. Immunohistochemistry was performed with 5% goat serum/PBS++ and primary antibodies, anti-PV (1:1000, Sigma Aldrich, St. Louis, MO, 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 hippocampal cornus ammonis (hippocampal CA) and medial frontal cortex (mFC) densities of GAD67GFP+ cells and PV+ cells were calculated using optical fractionator approach and unbiased counting rules with 3-dimensional 150 × 100 × 10 μm counting frames, on a 450 × 450 μm grid for mFC and 600 x 600 μm grid for hippocampal CA, using a 40× objective lens (Stereoinvestigator; MBF Biosciences). Stereological counting to determine cell density, displayed as means and standard errors of the mean, was performed in 3–8 serial coronal sections (every 10th section) of the adult mFC and the hippocampal CA as previously described (Stevens et al., 2013; Lussier and Stevens, 2016; Gumusoglu et al., 2017b). GAD67GFP+ and PV+ cells in coronal tissue sections was measured using fluorescent microscopy with a Zeiss Axioimager M2 microscope.
Data analysis and sampling

For embryonic assays, unless otherwise noted, all experiments were conducted with equal sample sizes of male and female embryos and sex genotyping was performed using PCR. For each assay, male and female outcomes were first compared separately for sex differences. If no sex differences were found, samples were pooled and sex-balanced sample sizes were reported. The impact of prenatal stress, hydrogen peroxide, or rotenone on migration and/or gene expression were evaluated using independent or paired sample t-tests where appropriate. ANOVA was used to evaluate the effects of prenatal stress and each antioxidant manipulation independently for each assay, looking for interactions that would indicate the ability of antioxidant treatment manipulation to significantly modify the main effect of prenatal stress. When appropriate, we performed post-hoc Bonferroni tests correcting for multiple comparisons.

For adult offspring behavior, a priori t-tests were used to assess any baseline differences between the control non-stressed and control prenatal stress groups (non-stress PBS vs. prenatal stress PBS) to determine the efficacy of any antioxidant rescue. ANOVAs were used to evaluate the effects of prenatal stress and each antioxidant manipulation independently for each assay, looking for interactions that would indicate the ability of antioxidant treatment manipulation to significantly modify the main effect of prenatal stress. When appropriate, I performed post-hoc Bonferroni tests correcting for multiple comparisons. Because of the relatively small sample sizes of some of the groups, trending significant values of $p < 0.07$ were reported in a few cases.
CHAPTER 3. AIM 1 RESULTS AND CONCLUSIONS

The goal of Aim 1 is to examine the effects of redox dysregulation and prenatal stress on the migration of GABAergic progenitor cells in the embryonic brain and to determine whether prenatal stress effects on the developing brain depend on redox dysregulation on the developing brain. I hypothesize that elevated levels of ROS--either through exogenous pro-oxidant exposure or prenatal stress-induced ROS--can be measured in the embryonic brain and will cause a delay in the migration of GABAergic interneuron progenitor cells into the developing neocortex. I also hypothesize that maternal pretreatment with antioxidants will ameliorate the delay in migration caused by prenatal stress.

Graphical abstract
Results for Aim 1.1: Hydrogen peroxide caused increased velocity and greater deviation from the migration stream of GABAergic progenitor cells

GAD67GFP+ cell movements were captured in vitro during migration into the developing neocortex of E14 embryo forebrain sections using live imaging (Fig. 3A-C). Matched forebrain sections showed that normal tangential interneuron migration (Fig. 3D-G) was disrupted by hydrogen peroxide (H$_2$O$_2$; Fig. 3H-K; equivalent results for 1 and 5 μM H$_2$O$_2$, so data were pooled). H$_2$O$_2$ caused GAD67GFP+ cells to deviate in their direction of migration compared to control. The angle of deviation of cell movement in control tissue measured from the direction of the tangent ranged from 0 to 170 deg, with the average cell movement in single sections ranging 20.5-53 deg (Fig. 3D-G, M). The angle of deviation in H$_2$O$_2$-exposed interneurons ranged from 0 to 180 deg, with the average cell movement in single sections ranging 54-96.5 deg ($p < 0.01$ from control; Fig. 3H-K, M). While the direction of cell movement was divergent with H$_2$O$_2$, the velocity of migrating GAD67GFP+ cells was also increased, from an average velocity across all samples of 41.7 μm/hr to 72.2 μm/hr—a 71.2% increase ($p < 0.01$; Fig. 3L). Cellular velocity ranged from 6.3 to 84 μm/hr in control conditions and 29.4 to 122.4 μm/hr with H$_2$O$_2$ exposure. Together, a model of these altered movements shows an overall delay in the population migration in the appropriate direction (Fig. 3N).

Results for Aim 1.2: Rotenone increased DHE oxidation in the embryonic brain and delayed migration of GABAergic progenitor cells

Intracerebroventricular rotenone injections were utilized to create a pro-oxidant environment in the embryonic brain in utero, confirmed by assessing a proxy for superoxide
levels, dihydroethidium (DHE) fluorescent intensity, in ganglionic eminence (Fig. 4A-B, G). Injections of rotenone into the ventricle of E13 embryonic brain, which generates ROS by inhibiting Complex I of the electron transport chain, resulted in increased DHE mean fluorescent intensity (MFI) in embryonic ganglionic eminence by 52.9% ($p < 0.05$) compared to saline control (Fig. 4A-B, G). Antimycin-A, which generates excess superoxide as a positive control, demonstrated that potential DHE MFI in control brains was 60.6% higher, similar to rotenone exposed samples ($p < 0.01$, data not shown). We further assessed the impact of this redox dysregulation on interneuron migration in utero. Rotenone ICV injections resulted in delays to the leading edge of migrating GABAergic neurons 24 hours later compared to saline injected controls ($p < 0.05$; Fig. 4E-F, H). The delay of GABAergic neurons recapitulates the migration delay at E14 after prenatal stress ($p < 0.05$; Fig. 4C-D, H) as shown previously (Stevens et al., 2013).

**Results for Aim 1.3: Prenatal stress altered redox gene expression, disrupted glutathione stores, dysregulated antioxidant enzyme activity, and increased ROS in the embryonic brain**

**Prenatal stress altered the expression of key genes involved in redox regulation.** We assessed the expression of genes involved in redox regulation in E13 brain after prenatal stress at E12, including those for antioxidant defense, *Gpx1, Cat, Trl, Sod1, Ses1, Ses2*, and *Ses3*, mitochondrial proteins involved in generating ROS, *Idh2, Aifm1, and Ndufs4*, and the antioxidant response factor *Nrf2*. (Fig. 5). After adjusting for multiple comparisons, prenatal stress increased gene expression of the antioxidant defense gene, *Trl* ($p < 0.05$; Fig. 5A) and increased expression of a gene that contributes to ROS production, *Aifm1* ($p < 0.01$; Fig. 5I). Changes in
these genes may have been downstream of increased expression of Nrf2 after prenatal stress ($p < 0.01$; Fig. 5H) and together may be linked with a dysregulated redox environment.

**Prenatal stress dysregulated glutathione stores and antioxidant enzyme activity.** To further assess embryonic brain changes as a result of prenatal stress (Stevens et al., 2013), we assessed embryonic brain for thiol states, indicative of redox conditions. Prenatal stress trended to increase the level of reduced to oxidized glutathione (GSH:GSSG) in the embryonic forebrain, compared to the non-stressed condition ($p = 0.05$; Fig. 6A). There was also no significant difference in reduced GSH levels in control versus prenatally-stressed embryonic forebrain (Fig. 6B). However, prenatal stress significantly decreased glutathione disulfide, GSSG ($p < 0.01$; Fig. 6C), suggesting a lack of antioxidant enzyme (glutathione peroxidase, GPx1) capacity in embryonic brain. Indeed, we found a trend towards a decrease in GPx1 activity after prenatal stress ($p = 0.06$; Fig. 6D). Prenatal stress also led to a trend 23.7% decrease in thioredoxin reductase activity ($p = 0.06$; Fig. 6E).

Because of these changes, we further assessed whether prenatal stress effects on embryonic brain redox dysregulation would be influenced by maternal N-acetylcysteine (NAC) administration, which restores intracellular glutathione and cysteine (Olsson et al., 1988). A two-way ANOVA revealed significant main effects of NAC ($F[1, 24] = 10.13, p < 0.01$) and an interaction of NAC with prenatal stress effects ($F[1, 24] = 4.66, p < 0.05$) on the reduced to oxidized glutathione (GSH:GSSG) ratio in the embryonic forebrain (Fig. 6A). NAC treatment also increased levels of reduced glutathione ($F[1, 25] = 4.31, p < 0.05$) (Fig. 6B) and decreased oxidized GSSG levels (main effect of NAC: $F[1, 24] = 12.10, p < 0.01$, NAC, stress interaction: $F[1, 24] = 5.91, p < 0.05$) (Fig. 6C). In general, maternal NAC had dramatic impacts on the
critical glutathione components of redox regulation and also prevented any additional dysregulation of glutathione stores in prenatally-stressed embryonic brain compared to control.

Maternal NAC also significantly increased GPx1 enzyme activity level (main effect: $F[1, 22] = 357.20, p < 0.01$) (Fig. 6D). Both non-stressed and prenatally-stressed embryos had increased GPx1 activity (174.9% increase, $p < 0.01$ and 231.5% increase $p < 0.01$ respectively; Fig. 6D), with prenatal stress no longer showing a significant decrease in activity compared to non-stressed embryonic brain in the presence of NAC.

**Prenatal stress increased levels of ROS in embryonic brain.** Prenatal stress influenced not only antioxidant enzymes but also shifted redox balance enough to alter levels of DHE oxidation in embryonic GABAergic progenitors, as assessed by DHE MFI in ganglionic eminence. Antimycin-A increased MFI as predicted in non-stressed control brains as compared to control (a prior t-test, $p < 0.01$; Fig. 7G). The non-parametric test, Kruskal-Wallis, showed that there was a statistically significant difference in DHE MFI between the prenatally-stressed and non-stressed NAC embryos and the prenatally-stressed and non-stressed PBS embryos ($H[3] = 18.56, p < 0.01$, Fig. 7H), with a mean rank MFI value of 19.5 for non-stressed PBS (Fig. 7A), 33.8 for prenatally-stressed PBS (Fig. 7B), 14.4 for non-stressed NAC (Fig. 7C), and 15.0 for prenatally-stressed NAC (Fig. 7D). Post-hoc tests of Dunn’s multiple comparisons test found differences in the MFI of non-stressed and prenatally-stressed PBS embryos ($p < 0.05$), prenatally-stressed PBS and non-stressed NAC embryos ($p < 0.01$), and prenatally-stressed PBS and prenatally-stressed NAC embryos ($p < 0.01$). The Kruskal-Wallis test also showed a significant difference in MFI between the prenatally-stressed and non-stressed AST embryos and the prenatally-stressed and non-stressed PBS embryos ($H[3] = 19.99, p < 0.01$, Fig. 7H), with a mean rank MFI value of 19.5 for non-stressed PBS (Fig. 7A), 34.3 for prenatally-stressed PBS
Post-hoc tests of Dunn’s multiple comparisons test found differences in the MFI of non-stressed and prenatally-stressed PBS embryos ($p < 0.05$), prenatally-stressed PBS and non-stressed AST embryos ($p < 0.01$), and prenatally-stressed PBS and prenatally-stressed AST embryos ($p < 0.01$).

**Results for Aim 1.4: Delays in GABAergic progenitor cell migration after prenatal stress normalized by maternal antioxidant**

As reported previously, PS delayed the migration of GABAergic progenitor cells into the developing neocortex at E13 and E14 (Fig. 8, (Stevens et al., 2013). Here, we replicated a delay after prenatal stress, compared to both non-stressed ($p < 0.05$, Fig. 8A and 8D) and maternal saline control ($p < 0.01$, Fig. 8G) conditions.

Since redox dysregulation disrupted GABAergic progenitor migration and prenatal stress induced redox dysregulation, we tested the hypothesis that the delay after prenatal stress was due to redox shifts. We increased maternal antioxidant status and found accelerated GABAergic progenitor migration. Maternal NAC ($F[1, 34] = 10.72, p < 0.01$; Fig. 8B and 8E) and AST ($F[1, 34] = 18.60, p < 0.01$; Fig. 8C and 8F) significantly increased migration, also interacting with prenatal stress that resulted in increased migration (NAC: $F[1, 34] = 8.96, p < 0.01$). Indeed, the extent of migration after prenatal stress differed significantly with and without either maternal NAC or AST (post-hoc, $p < 0.01$).

Prenatal stress also trend-wise decreased expression of a chemoreceptive gene involved in migration, $Cxcr4$, (Sanchez-Alcaniz et al., 2011) in GABAergic progenitors ($p = 0.06$; Fig. 8H). $Cxcr4$ expression in the presence of maternal NAC was not affected by prenatal stress,
although expression after prenatal stress was not significantly different dependent on NAC condition.

**Aim 1 conclusions and discussion**

Redox dysregulation is a mediator of the impact of multiple environmental exposures, suggesting that it may also play a role in mediating the effects of in utero environmental changes like prenatal stress (Wells et al., 2009). The balance between oxidative and reductive processes is critical during rapid periods of cell growth and differentiation, so even small changes in reactive oxygen species (ROS) during embryonic brain development could have significant effects (Dennery, 2007). I show here that redox dysregulation influences interneuron migration and that prenatal stress leads to redox dysregulation in the embryonic brain. I also show that manipulation of redox balance (in this case, maternal administration of antioxidants) interacts with prenatal stress effects on embryonic brain and can prevent both the increased level of ROS and migration delay. These findings suggest a biochemical pathway that could be targeted to prevent altered neurodevelopment exemplified by prenatal stress effects.

Direct exposure of embryonic brain to the pro-oxidant, hydrogen peroxide (H₂O₂), results in deviations of cellular direction and increased velocity (Fig. 3L-M). Although H₂O₂ is not a free radical by definition, it can form hydroxyl radicals through the Fenton reaction with Fe²⁺ and is detoxified by classic antioxidants (Burton and Jauniaux, 2011). This increased rate of movement in multidirectional, non-tangential pathways could result in an overall delay in migration as seen in offspring after prenatal stress (Stevens et al., 2013). ROS may be strongly connected to mitochondrial dysfunction including oxidative phosphorylation (Burton and Jauniaux, 2011), disruptions of which alter migratory trajectory (Lin-Hendel et al., 2016). Lin-
Hendel et al. (2016) found migration patterns of cortical interneurons with dysfunctional mitochondria exhibited more frequent and aberrant directional changes and slower migratory rates. The increase in multidirectional migration patterns that we found here may suggest a mitochondrial redox imbalance induced by H$_2$O$_2$. Our findings that migration was also delayed by *in utero* intracerebroventricular injection of rotenone (Fig. 4) also support a central role for mitochondrial redox regulation in the movements of this progenitor cell population.

The impact of prenatal stress on interneuron migration (Stevens et al., 2013) may involve redox effects on the embryonic brain. In support of this hypothesis, we found changes in the expression of redox-related genes in embryonic brain after prenatal stress (Fig. 5). Increased expression of thioredoxin reductase (*Tr1*) suggests a compensatory response to oxidative stress. Elevated nuclear factor erythroid-derived 2 (*Nrf2*) levels demonstrate that embryonic neuronal precursors *in vivo* have a concerted response to maternal stress to induce genes with antioxidant response elements to which *Nrf2* binds. Increased expression of apoptosis-inducing factor 1, mitochondrial (*Aifm1*) is critical for normal mitochondrial function and may indicate that a subset of the embryonic progenitors assessed had greater energy metabolism after prenatal stress and may have generated more ROS in the process.

These gene expression findings suggest that the embryonic brain was in a more pro-oxidant state after prenatal stress. However, glutathione redox balance did not demonstrate increases in GSSG. Reduced glutathione (GSH) is an important antioxidant that in combination with GPx1 activity directly reduces hydroperoxides, in turn producing glutathione disulfide (GSSG). As such, the ratio of oxidized to reduced form (GSH:GSSG) is used as a marker of redox dysregulation, or oxidative stress (Zitka et al., 2012). The decreased levels of GSSG we found after prenatal stress suggest that embryonic brain had lower levels of oxidative stress.
When taken with our data that shows lower GPx1 enzyme activity level as a result of prenatal stress (Fig. 6D), there appears to be a deficiency of this antioxidant pathway induced by prenatal stress. In the absence of sufficient GPx1 activity, glutathione levels (GSH and GSSG) may shift to a more reduced state. The influence of NAC on glutathione peroxidase activity corrected the deficit of activity found with prenatal stress and increased overall glutathione levels as expected. In the presence of maternal NAC, prenatal stress did not alter glutathione levels as it did in the absence of NAC (Fig. 6).

Interestingly, TR and redox states are interdependent (Perez-Torres et al., 2017). Despite an increase in TR enzyme gene expression with prenatal stress (Fig. 5A), its activity level trended downwards (Fig. 6E) which may result from glutathionylation facilitated by an oxidative state of the cell (Casagrande et al., 2002). Reactive oxygen species may have arisen locally but also may have been contributed from the maternal-placental unit in a diffusible form such as oxidized lipids.

N-acetyl cysteine (NAC) is an aminothiol and synthetic precursor of intracellular GSH (van Zandwijk, 1995). When given to pregnant dams, ROS levels abnormally increased by prenatal stress were normalized (Fig. 7D), indicated by DHE oxidation, measured by dihydroethidium (DHE). Under normal physiological conditions, the most ubiquitous oxygen free radical is the superoxide anion. Superoxide is detoxified by the superoxide dismutase (SOD) enzyme, which converts it to hydrogen peroxide (a less reactive molecule). When given simultaneously with restraint stress, astaxanthin (AST) also normalized DHE oxidation in the brain (Fig. 7F). AST, a xanthophyll carotenoid, is a potent antioxidant that is known to cross the blood brain barrier and scavenge ROS. It possesses neuroprotective properties by enhancing the antioxidant enzymes SOD and GPx1 and reducing lipid peroxidation (Liu and Osawa, 2009; Wu...
et al., 2014; Al-Amin et al., 2016a). Known to cross the placenta, NAC works to restore GSH and may be able to cross the blood brain barrier (Horowitz et al., 1997; Bavarsad Shahripour et al., 2014). Nonetheless, the ability of GSH to quench superoxide radicals has been demonstrated before (Heribert and Helmut, 1983). NAC and AST may both act either directly on embryonic brain by potentially crossing the placenta or through altering maternal redox balance which secondarily impacts embryonic brain. These findings demonstrate that maternal redox manipulation and maternal stress interact in previously undiscovered ways to influence embryonic brain development.

The influence of maternal states on embryonic brain redox balance is critical, given the experiments here that are the first to show that direct redox dysregulation of embryonic brain disrupts interneuron migration. Here, we replicated our previous findings showing that prenatal stress influences interneuron migration (Stevens et al., 2013) (Fig. 8) and applied the method of maternal redox manipulation to test its efficacy in preventing these effects. Offspring of mothers pretreated with NAC or AST showed normalized interneuron migration despite prenatal stress (Fig. 8E and 8F, respectively). These findings are in line with the impact of high levels of oxidative radicals on radial migration in the developing forebrain (Narasimhaiah et al., 2005), although prenatal stress does not induce apoptosis in migrating interneurons (Stevens et al., 2013). The mechanism by which pro-oxidant states impact interneuron migration may involve their chemoreceptive properties, such as Cxcr4 expression. Cxcr4 is influenced by the repressor Yin Yang 1 which is sensitive to pro-oxidant states (Hasegawa et al., 2001; Beck et al., 2010). However, there may also be non-transcriptionally-mediated impacts of redox dysregulation on mitochondrial function which is critical to cell migration. These findings suggest an important mechanism by which environmental exposures may influence neurodevelopment that may
converge with genetic risk such as 22q11 mutations that influence the same cellular process (Meechan et al., 2012).

Indirect interaction of maternal redox manipulation with prenatal stress, rather than direct redox impacts on embryonic brain, may be responsible for the effects we found here. However, we observed significant main effects of NAC and AST treatment, both on DHE oxidation levels in embryonic brain and on extent of migration (Fig. 7 and 8), further demonstrating the sensitivity of neuronal precursors generally and cortical interneuron migration specifically to redox regulation.

To my knowledge, these experiments are the first to investigate the role of dysregulated redox states as an intermediary between the effects of prenatal stress and alterations in embryonic brain. Numerous studies have shown that redox dysregulation may be a component of physiological stressors that occur during pregnancy (Peuchant et al., 2004; Cambonie et al., 2007b; Derks et al., 2010a; Ziech et al., 2011). Targeting an appropriate balance of redox biology during neurodevelopment may have benefits for offspring brain and overall development. In particular, these findings may be applicable to elucidating poorly understood mechanisms of early contributions to mental illness, including genetic, biological, and environmental causes, and ultimately developing better treatments and interventions for those at risk for neuropsychiatric illness.
Figure 3. Hydrogen peroxide caused increased velocity and greater deviation from the migration stream of GABAergic progenitor cells.

(A-C) Arrow indicates direction of migrating GABAergic progenitor cells and arrowheads indicate the starting position and end position of interneuron after 30 minutes. (A) Tissue section of E14 embryo in artificial cerebral spinal fluid (aCSF) at beginning time point (t = 0 minutes).
Figure 3 – continued. (B) Tissue section pictured in A at t = 30 minutes. (C) Magnification of A and B with mash-up of time points 0 and 30 minutes. Schematic of migration of 10 interneuron progenitors after approximately 1-1.5 hours of E14 embryo in aCSF (D-G) and aCSF with either 1μM H2O2 (H-J) or 5μM H2O2 (K). Arrow indicates direction of migration stream and dot is end-point. (L) Bar graph shows average velocity of GAD67GFP+ cells and (M) depicts average degree of deviation from migration stream. (N) Schematic of hypothesized migration delay showing average velocity and average angle deviation for each of the five live imaging sessions. For the purpose of calculating migration deviation, we designated interneurons confined to migration stream as deviating 0°. Dashed lines represent the boundaries of the tissue slices. (**) p < 0.01 by two-tailed paired t-test, n = 5 embryos, aCSF and H2O2 groups)
ICV-injected rotenone increased levels of DHE oxidation in E14 primordial ganglionic eminence (GE) tissue compared to ICV-injected saline, as measured by dihydroethidium (DHE) mean fluorescent intensity (MFI) of cells and normalized to MFI = 1.00 (A-B, G). Prenatal stress (PS) delays progenitor migration at E14 (D) compared to non-stressed (C) embryonic neocortical tissue (H). ICV-injected rotenone (F) also delays migration of GAD67GFP+ cells at E14 compared to ICV-injected saline (E, H). For the DHE experiments, ICV Saline: n = 5,
Figure 4 – continued. and ICV Rotenone: n = 5. For the migration experiments, NS: n = 10, PS: n = 4, ICV Saline: n = 8, and ICV Rotenone: n = 8. (* $p < 0.05$ compared to ICV saline by paired t-test, $p < 0.05$ compared to NS by paired t-test)
Antioxidant activity gene, *Tr1* (A-G), as well as other genes related to redox regulation (H-K) were altered. NS = non-stressed; PS = prenatal stress; NS: n = 20, PS: n = 20; *p < 0.05, **p < 0.01 by two-tailed independent t-tests controlling for multiple comparisons by Bonferroni
Figure 6. PS disrupted glutathione stores and dysregulated antioxidant enzyme activity.

(A) Ratio of reduced glutathione (GSH) to glutathione disulfide (GSSG) showed an increase as the result of prenatal stress (vs. non-stress [NS], $p = 0.05$), but showed a main effect of NAC treatment (## $p < 0.01$ by two-way ANOVA) and an interaction of stress and treatment ($\phi p < 0.01$).
Figure 6 – continued. (B) A main effect of NAC (# $p < 0.05$) on GSH. (C) A significant baseline difference was observed between non-stress and prenatal stress control brains (** $p < 0.01$), and main effects of NAC (## $p < 0.01$) and stress (α $p < 0.05$), as well as an interaction of stress and NAC ($ϕ p < 0.05$) was found in GSSG brains. (D) A trend decrease in GPx1 activity resulting from prenatal stress (vs. NS, $p = 0.06$) and a main effect of NAC (## $p < 0.01$) was detected. (E) A trend decrease in TR activity was found between NS and PS embryonic forebrains ($p = 0.06$). For glutathione experiments, NS: n = 6, PS: n = 6, NS NAC: n = 10, and PS NAC: n = 7. For GPx1 experiment, NS: n = 12, PS: n = 14, NS NAC: n = 3, and PS NAC: n = 3. For TR experiment, NS: n = 12, and PS: n = 14.
Figure 7. PS increased levels of DHE oxidation in the primordial ganglionic eminence of the embryonic brain and maternal antioxidants abrogated the increase.

(A) All analyses were normalized to mean fluorescent intensity, MFI = 1.00 of control (Con) tissue. Measurements of MFI were taken with 40x objective images. (B) PS increased MFI
Figure 7 – continued. ($p < 0.05$ compared to control using post-hoc test). In the absence of PS, MFI in NS NAC (C) and NS AST (E) were comparable to levels of control. PS NAC (D) and PS AST (F) decreased levels of MFI (**$p < 0.01$ compared to PS using post-hoc test). (G) Antimycin-A (AA) was used as the positive control and showed the highest DHE oxidation ($p < 0.05$ compared to control using a priori t-test).
Figure 8. Delays in GABAergic progenitor migration after PS normalized by maternal antioxidants.

(A) NS control migration, (B) NS NAC migration, and (C) NS AST migration. (D) PS control migration was significantly delayed compared to NS control ($p < 0.05$ by independent t-test) and NS saline (micrograph not shown; quantitative data shown in G). (E) PS NAC migration was significantly increased compared to PS (** $p < 0.01$) and a main effect of NAC (## $p < 0.01$) was revealed. (F) PS AST migration was significantly increased compared to PS control (*** $p < 0.01$)
Figure 8 – continued. and a main effect of AST (## \( p < 0.01 \)) was found. A main effect of stress (\( \alpha \) \( p < 0.05 \)) was also unveiled between PS embryos (D, E, and F) and NS embryos compared to (A, B, and C). The interaction of stress and NAC treatment (\( \phi \phi \) \( p < 0.01 \)) was also significant (G). (H) Quantitative PCR of the Cxcr4 gene, coding for a receptor expressed by migrating inhibitory progenitor cells (\( p = 0.06 \)). Two different two-way ANOVAs were performed to measure the effects of NAC and AST compared to control embryonic brains.
CHAPTER 4. AIM 2 RESULTS AND CONCLUSIONS

The goal of Aim 2 is to examine the effects of redox dysregulation and prenatal stress on adult offspring behavior and postnatal GABAergic neuron populations in adult offspring brain. This Aim serves to test adult offspring mice for: 1) an anxiety-like phenotype using the open-field test and the elevated plus maze, 2) social approach in the three-chamber sociability and social recognition task, and 3) behaviors in the sensorimotor domain using the open field test, rotarod performance test, water T-maze task, and pre-pulse inhibition test. I hypothesize that prenatal stress-mediated redox dysregulation in the embryonic brain during development will have lasting effects on adult offspring behavioral outcomes. Specifically, I hypothesize that prenatal stress control mice will exhibit a more anxious-like phenotype, display less sociability, and perform more poorly on the sensorimotor tasks than the non-stressed offspring mice. I hypothesize that NAC and AST will rescue these behavioral deficits—maternal antioxidant treatment will restore behavior to levels of the non-stressed control mice. I also hypothesize that the proportion of PV+ and GAD67+ neuron populations will be lower in the adult brain and maternal antioxidant treatments during prenatal stress will assuage oxidative alterations in the embryonic brain and prevent changes to cell densities in the adult brain.
Results for Aim 2.1: Prenatal stress increased anxiety-like behavior in male and female offspring and maternal antioxidant treatments prevented this phenotype

Open-field test (OFT)

In order to assess an anxiety-like phenotype in adult offspring mice, male and female mice underwent testing in the OFT (Fig. 9). No baseline differences in the first five minutes center time were detected between prenatally-stressed and non-stressed control (PBS) male mice and no main effect of prenatal stress was found (Fig. 9A). However, both maternal antioxidant treatments, N-acetylcysteine (NAC) and astaxanthin (AST), significantly increased the amount of time male mice spent in the center of the open field on Day 1 of testing, in the first 5 minutes (NAC: $F[1, 41] = 9.44, p < 0.01$; AST: $F[1, 40] = 5.57, p < 0.05$; Fig. 9A). This was driven by the following differences: PS NAC male mice spent 127.0% more time in the center and PS AST male mice spent 143.0% more time in center of the open field test in the first 5 minutes compared to the PS PBS male mice (post-hoc, $p < 0.05$ and $p < 0.05$, respectively), suggesting that prenatally-stressed male offspring were more susceptible to the reduction in anxiety-like behavior from maternal antioxidant treatments.

No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) female mice and no main effect of stress was found. Neither NAC nor AST had significant effects on the center time of female mice in the first 5 minutes of the open field test (Fig. 9B).
Elevated plus maze (EPM)

Examining the amount of time a mouse spends in the different zones of the EPM is another important indicator of an anxiety-like phenotype in mice. Prenatally-stressed mice have been shown to spend more time in the closed arms of the EPM compared to their non-stressed counterparts (Lussier and Stevens, 2016). Here, a priori t-test found that PS PBS male mice spent more time in the closed arm in the EPM, suggesting an anxious-like phenotype, compared to the NS PBS male mice ($p < 0.05$, Fig. 10A). Prenatally-stressed mice spent more time in the closed arm of the EPM than non-stressed mice across NAC treatment groups (two-way ANOVA testing prenatal stress and NAC treatment $F [1, 44] = 8.18, p < 0.05$; Fig. 10A). An interaction of maternal AST treatment with prenatal stress was found in the increased amount of time spent in the closed arm of the EPM, demonstrating that in the presence of AST, prenatal stress did not increase anxiety-like behavior ($F [1, 43] = 5.38, p < 0.05$; Fig. 10A). A ratio of time spent in the open to closed arm of the EPM was calculated. Prenatal stress significantly decreased the ratio of time spent in the open to closed arm of the EPM for the male mice across NAC treatment groups (two-way ANOVA testing prenatal stress and NAC treatment: $F [1, 42] = 4.61, p < 0.05$; Fig. 10B). While no significant interactions of stress with antioxidant treatment were found, this decrease in the ratio with maternal stress was not observed in the AST treatment conditions, suggesting that the maternal AST treatments during pregnancy normalized EPM anxiety-like behavior in non-stressed compared to prenatally-stressed male offspring (Fig. 10B). The number of entries into the open and closed arms of the EPM was also assessed. The ratio of open:closed arm entries by the mice was significantly decreased among the prenatally-stressed male mice across NAC treatment groups (two-way ANOVA testing stress and NAC treatment: $F [1, 41] = $
5.55, \( p < 0.05 \); Fig. 10C), indicating that prenatal stress increased anxiety-like behavior and NAC did not change the effects. The lack of interaction and prenatal stress effects in the AST group suggests that in the presence of AST, prenatal stress did not have the same effect on anxiety-like behavior (Fig. 10C).

A priori t-tests of amount of time spent in the closed arm of the EPM and the ratio of time spent in the open:closed arm did not reveal any differences between the non-stressed and prenatally-stressed control (PBS) female mice. However, the open:closed arm entries ratio found that prenatally-stressed female control mice (PBS) had fewer open arm than closed arm entries than non-stress females (a priori t-test, \( p < 0.05 \), Fig. 10F), suggesting a more anxious-like phenotype at baseline. Main effects of prenatal stress across PBS and antioxidant groups were also detected. Prenatally-stressed female mice spent less time in the open compared to closed arms of the EPM (two-way ANOVA testing stress and AST treatment: \( F[1, 41] = 4.92, p < 0.05 \); Fig. 10E) and had fewer entries into the open arms of the EPM compared to the closed arms (two-way ANOVA testing stress and antioxidants treatments, NAC: \( F[1, 42] = 7.47, p < 0.01 \); AST: \( F[1, 40] = 6.70, p < 0.05 \); Fig. 10F). AST treatment, but not NAC treatment, increased the ratio of open:closed arm entries (\( F[1, 40] = 5.68, p < 0.05 \); Fig. 10F). Further examination of this effect suggests that AST, but not NAC, ameliorated the anxious-like phenotype observed in the prenatally-stressed female mice. However, a post-hoc t-test found that NS AST mice spent significantly more time in the open arm of the EPM than the PS AST mice (\( p < 0.05 \), Fig. 10E), suggesting an impact on offspring from AST treatment alone.
Results for Aim 2.2: Prenatally-stressed female mice, but not male mice, showed a deficit in sociability that was not rescued by either maternal antioxidant treatment. Prenatal stress had no effect on preference for social novelty in either sex.

Three chamber sociability task

Male and female offspring mice were also subjected to the three-chamber sociability and social recognition test. No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) male mice (Fig. 11A), but a main effect of stress was found across NAC and PBS groups. Prenatally-stressed male mice spent significantly less time with the empty cup (two-way ANOVA testing prenatal stress and NAC treatments: $F[1, 43] = 5.72, p < 0.05$; Fig. 11A), but no more time with the mouse cup (two-way ANOVA testing prenatal stress and NAC treatments: $F[1, 44] = 0.00, p = 0.98$; Fig. 11B). No significant effects across PBS and AST groups were found, suggesting that prenatal stress did not have the same effect in the presence of AST treatment. In time spent with the mouse cup compared to the empty cup, a sociability measure, as expressed by the ratio mouse:empty cup, male mice across all conditions showed no group differences, indicating no preference for either another mouse or an inanimate object (Fig. 11C).

No baseline differences were detected between prenatally-stressed and non-stressed control (PBS) female mice among any of the three measures (11D-F). An interaction of either maternal antioxidant with prenatal stress was found in the amount of time spent near the empty cup, having the effect of maternal antioxidants returning the amount of time spent near the empty cup to levels similar to the non-stressed PBS mice (NAC: $F[1, 43] = 4.62, p < 0.05$ and AST: $F[1, 43] = 5.16, p < 0.05$; Fig. 11D). Prenatally-stressed AST mice, but not prenatally-stressed
NAC mice, spent less time with the empty cup than the prenatally-stressed PBS mice (post-hoc, \( p < 0.05 \); Fig. 11D). Also, a trending significant main effect of prenatal stress was present with prenatally-stressed mice spending less time with the mouse cup across PBS and AST groups (two-way ANOVA testing prenatal stress and AST treatment: trend sig., \( F[1, 42] = 3.96, p = 0.05 \); Fig. 11E), with a post-hoc test revealing that prenatally-stressed AST female mice spent less time with the mouse cup than non-stressed AST mice (\( p < 0.05 \), Fig. 11E). These findings together suggest that prenatal stress, in the presence of maternal AST exposure, reduced exploration across different domains in female offspring. A calculated sociability ratio of time spent with the mouse:empty cup did not reveal any differences among the conditions of female mice, suggesting that the impact of prenatal stress and antioxidants on sociability were not remarkable when overall animal exploration of either cup was considered.

Three chamber social recognition task

In agreement with previous findings (Lussier and Stevens, 2016), no baseline differences were detected in prenatally-stressed and non-stressed control (PBS) male mice, and no main effect of prenatal stress was found in social recognition (Fig. 12). Male mice across groups showed no difference in social recognition as indicated by no groups difference in the ratio of time spent in proximity of the novel:familiar mouse cups (Fig. 12C).

Similarly, no baseline differences in social recognition were detected in prenatally-stressed and non-stressed control (PBS) female mice, and no main effect of prenatal stress was found. Female mice across groups did not show group differences in social recognition in the amount of time spent in proximity of the novel:familiar mouse cups (Fig. 12F). AST female mice did, however, spend less time with the familiar mouse (\( F[1, 40] = 6.01, p < 0.05 \); Fig.
12D), but no more time with the stranger mouse (Fig. 12E). Maternal AST, therefore, reduced female offspring social interaction with a mouse previously encountered compared to control offspring, suggesting an enhancement of normal social down-regulation regardless of prenatal stress.

**Results for Aim 2.3:** Prenatal stress caused arrested motor learning on the rotarod in both sexes, deficits in sensorimotor gating on the PPI task in males, and increased cognitive flexibility on the water T-maze in males. Antioxidant treatments prevented some of the behavioral deficits observed in the mice.

**Prepulse inhibition (PPI) of the acoustic startle reflex**

**Pulse alone.** No baseline differences in response to pulse alone were detected in prenatally-stressed and non-stressed control (PBS) male mice, and no main effect of prenatal stress was found (Fig. 13A). As expected, the startle reflex response in PBS, NAC, and AST mice was not significantly different in the male offspring mice when a pulse alone was administered (Fig. 13A).

No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) female mice. The baseline startle reflex response in PBS, NAC, and AST mice was not significantly different in the female offspring mice when a pulse alone was administered (Fig. 13B).

**Across all decibel (dB) levels.** 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). A priori ANOVA found that prenatally-stressed male control mice (PBS) showed deficits in inhibiting their startle response compared to non-stress control mice across the three dB levels \( F[1, 62] = 4.77, p < 0.05, \) Fig. 13C). PBS mice also showed significant improvement in gating their startle reflex with every incremental increase in dB level \( F[2, 62] = 12.30, p < 0.01, \) Fig. 13C). Specifically, PBS mice significantly improved their PPI between 5 dB and 10 dB \( (\text{post-hoc, } p < 0.01) \) and 5 dB and 15 dB \( (\text{post-hoc, } p < 0.01) \) levels. No differences were detected in NAC prenatally-stressed male mice and NAC non-stress mice across the three different dB levels \( F[1, 63] = 0.05, p = 0.83, \) Fig. 13C), suggesting that maternal NAC administration prevented the deficits of prenatal stress. Similar to PBS mice, though, NAC mice showed improvements in sensorimotor gating with increases in dB level \( F[2, 63] = 17.81, p < 0.01, \) Fig. 13C). NAC male mice significantly improved from 5 dB and 10 dB \( (\text{post-hoc, } p < 0.01) \) and 5 dB and 15 dB \( (\text{post-hoc, } p < 0.01) \) levels. Surprisingly, non-stressed male AST mice showed deficits in inhibiting their startle response across the three different dB levels compared to the prenatally-stressed AST mice \( F[1, 61] = 17.15, p < 0.01, \) Fig. 13C), obscuring whether AST was able to rescue the deficit due to prenatal stress. However, prenatal stress AST male offspring appeared to have control levels of performance. Unlike PBS and NAC mice, AST mice showed overall significant improvement with increases in dB level \( F[2, 61] = 7.60, p < 0.01, \) Fig. 13C), but not with every incremental increase. AST mice only improved their PPI between 5 dB and 10 dB \( (\text{post-hoc, } p < 0.01) \) and 5 dB and 15 dB \( (\text{post-hoc, } p < 0.01) \) levels.

No baseline differences in PPI between prenatal stress and non-stress condition was detected in the PBS control female mice \( F[1, 63] = 1.16, p = 0.21, \) Fig. 13D). PBS mice did, however, show significant improvement in gating their startle reflex with incremental increases.
in dB level ($F[2, 63] = 6.24, p < 0.01$, Fig. 13D). Specifically, PBS mice significantly improved their PPI scores between 5 dB and 10 dB (post-hoc, $p < 0.01$) and 5 dB and 15 dB (post-hoc, $p < 0.01$) levels. A significant interaction between prenatal stress and dB level in NAC-exposed females, with non-stressed NAC females performing poorly at 5 dB, suggested that NAC on its own (without the effects of prenatal stress) caused female mice to perform poorly on this aspect of the PPI test ($F[2, 63] = 4.07, p < 0.05$, Fig. 13D). However, like PBS mice, NAC female mice showed improvement across the 5, 10, and 15 dB levels ($F[2, 63] = 13.07, p < 0.01$, Fig. 13D). NAC mice significantly improved their startle reflex inhibition between 5 dB and 10 dB (post-hoc, $p < 0.01$) and 5 dB and 15 dB (post-hoc, $p < 0.01$) levels. Prenatal stress had no significant impact on PPI in AST female mice, and unlike PBS and NAC mice, AST mice showed no improvements in their PPI with incremental increases in dB levels (Fig. 13D).

Differences across groups were also assessed at each intensity level to examine main effects and interactions of prenatal stress and antioxidant exposure:

**5 dB.** No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) male mice and no main effect of prenatal stress was found at the 5 dB level (Fig. 13C). Two-way ANOVA revealed an interaction of AST with prenatal stress effects on the PPI% at 5 dB of male mice ($F[1, 42] = 4.64, p < 0.05$, Fig. 13C). While prenatal stress effects and maternal antioxidants did not have significant main effects on PPI% overall at 5 dB, the interaction indicated that AST had the opposite effect on PPI% (compared to PBS) depending on whether the offspring experienced prenatal stress in utero.

In female mice, no baseline differences were detected in prenatally-stressed and non-stressed control (PBS) female mice and no main effect of prenatal stress was found at the 5 dB level (Fig. 13D). AST overall increased the sensorimotor gating ability of the mice ($F[1, 41] = 81$)
13.57, \( p < 0.01 \) as evidenced by increased PPI\% at 5 dB (Fig. 13D). An interaction of NAC and prenatal stress effects was also discovered (\( F[1, 42] = 10.52, p < 0.01 \)). This suggests that maternal antioxidants compared to PBS generally improved PPI in prenatally-stressed female mice (post-hoc, NAC: \( p < 0.05 \) and AST: \( p < 0.05 \), compared to prenatally-stressed PBS, Fig. 13D). However, non-stressed NAC females also had lower PPI\% (post-hoc, \( p < 0.01 \), compared to prenatally-stressed NAC), indicating deficits in their ability to reduce their startle reflex in response to the 5 dB prepulse stimulus.

10 dB. No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) male mice at the 10 dB level (Fig. 13C). Prenatal stress trend decreased the ability of male mice to dampen their startle response after the 10 dB prepulse stimulus across PBS and NAC groups (trend sig., two-way ANOVA testing prenatal stress and NAC treatments: \( F[1, 41] = 3.43, p = 0.07; \) Fig. 13C). An interaction of AST and prenatal stress effects was uncovered (\( F[1, 40] = 8.20, p < 0.01; \) Fig. 13C), indicating that prenatal stress increased the PPI\% of male mice when maternal treatment of AST was given, but prenatal stress caused deficits in PPI when PBS was given. This interaction clarifies that the main effect “improvement” of prenatal stress overall at 10 dB was driven by the AST group. Prenatally-stressed AST male mice showed a trend improvement in their sensorimotor gating reflex response compared to prenatally-stressed PBS mice (post-hoc, \( p = 0.07 \)) and a significant improvement compared to non-stressed AST (post-hoc, \( p < 0.05 \)). As with 5 dB results, this suggests prenatal stress male offspring had a benefit in PPI from AST but not NAC.

No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) female mice and no main effect of prenatal stress was found at the 10 dB level (Fig. 13D). No differences were uncovered in the performance at 10 dB of the AST female mice compared
NAC female mice, however, performed better on the PPI task than the PBS group as a whole ($F [1, 42] = 4.27, p < 0.05$; Fig. 13D), with prenatally-stressed NAC mice performing significantly better than the prenatally-stressed PBS mice (post-hoc, $p < 0.05$).

**15 dB.** No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) male mice and no main effect of prenatal stress was found at the 15 dB level (Fig. 13C). An interaction of AST and prenatal stress effects was revealed ($F [1, 41] = 8.80, p < 0.01$, Fig. 13C), indicating that when maternal treatment of AST was given, PPI% worsened in the non-stressed male mice, but improved in the prenatally-stressed male mice. This trend is the opposite direction of the PBS male mice. Non-stressed AST mice had lower PPI% than the non-stressed PBS mice (post-hoc, $p < 0.05$) and prenatally-stressed AST mice (post-hoc, $p < 0.01$).

No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) female mice and no main effect of prenatal stress was found at the 15 dB level (Fig. 13D). No differences were uncovered in the performance at 15 dB of the AST female mice compared to the PBS female mice. But, maternal antioxidant treatments of NAC improved the PPI% of female mice compared to PBS mice ($F [1, 42] = 4.23, p < 0.05$, Fig. 13D), suggesting again an improvement in performance overall from NAC exposure.

**Locomotor activity on the OFT on Day 2**

Day 2 in the OFT was used as a measurement of locomotor activity in the offspring mice, to avoid effects of environmental novelty in Day 1 measurements. No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) male mice, and no main effect of prenatal stress was found (Fig. 14A). An interaction of AST with prenatal stress effects (trend sig., $F [1, 41] = 3.61, p = 0.06$, Fig. 14A) was found in locomotion on Day 2, suggesting AST
may affect locomotor activity differently than PBS, depending on whether the mouse experienced prenatal stress.

No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) female mice and no main effect of prenatal stress was found (Fig. 14B). There was, however, an interaction of NAC with prenatal stress effects among the female mice ($F [1, 41] = 8.57, p < 0.01$, Fig. 14B). Non-stressed NAC female mice displayed increased locomotor activity compared to prenatally-stressed NAC mice (post-hoc, $p < 0.01$) and increased locomotion compared to non-stressed PBS mice (post-hoc, $p < 0.05$), indicating again that female offspring exposure to NAC alone can have significant impacts on behavior.

**Rotarod**

The rotarod performance task is an important measurement of motor coordination and motor learning. No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) male mice, but a main effect of prenatal stress was found (Fig. 15A-C). Prenatally-stressed mice had lower motor learning coefficients than non-stressed mice (three-way ANOVA testing for prenatal stress effects, antioxidant treatment effects, and day of trial effects; NAC: $F [1, 132] = 8.41, p < 0.01$ and AST: $F [1, 132] = 23.95, p < 0.01$). Also, all three treatment groups showed significant daily motor learning (NAC: $F [2, 132] = 9.87, p < 0.01$, and AST: $F [2, 132] = 8.91, p < 0.01$, Fig. 15A-C). Two separate, three-way ANOVAs revealed an interaction of maternal antioxidants, NAC and AST, with prenatal stress effects on the motor learning coefficients of male mice (NAC: $F [1, 132] = 5.23, p < 0.05$ and AST: $F [1, 132] = 18.93, p < 0.01$), generally suggesting that maternal antioxidants improved the motor learning of non-stressed animals, but had no effect on prenatally-stressed male mice. Maternally-treated
AST offspring displayed improvements in motor learning compared to PBS mice as evidenced by an AST treatment effect (F [1, 132] = 6.14, p < 0.05, Fig. 15A, C).

No baseline differences were detected in prenatally-stressed and non-stressed control (PBS) female mice, but a main effect of prenatal stress was found across NAC and PBS groups (Fig. 15D-F). Prenatally-stressed female mice had lower motor learning coefficients than non-stressed mice (three-way ANOVA testing for prenatal stress effects, NAC effects, and day of trial effects F [1, 132] = 26.27, p < 0.01). Similar to the male mice, female mice displayed significant daily motor learning across all the treatment groups (NAC: F [2, 132] = 3.56, p < 0.05, and AST: F [2, 132] = 7.76, p < 0.01, Fig. 15D-F). An interaction of NAC and prenatal stress effects was revealed (F [1, 132] = 13.02, p < 0.01; Fig. 15D-E), indicating that when maternal treatment of NAC was given, prenatal stress impacted female offspring differently than PBS. Maternally-treated AST female offspring overall showed enhancements in motor learning compared to PBS mice (F [1, 132] = 4.73, p < 0.05) (Fig. 15D, F).

**Water T-maze**

The water T-maze was performed on the offspring mice to assess habitual learning, as measured by time to reach the Trial Probe, and cognitive flexibility, as measured by time to reach the platform in the Reversal Trial. No baseline differences in habitual learning were detected in prenatally-stressed and non-stressed control (PBS) male mice. However, prenatally-stressed mice showed impairments in habitual learning compared to non-stress mice across PBS and AST groups (two-way ANOVA testing prenatal stress and AST treatments: F [1, 33] = 7.94, p < 0.01; Fig. 16A). A stress by AST interaction was also detected (F [1, 33] = 7.13, p < 0.01), indicating that AST treatment coupled with maternal prenatal stress drove the impaired habitual
learning of prenatally-stressed male mice, but control PBS mice habit learning was not affected by prenatal stress. Prenatally-stressed AST male mice, compared to prenatally-stressed PBS (post-hoc, $p < 0.01$) and non-stressed AST (post-hoc, $p < 0.01$), spent more time swimming to find the platform, suggesting impaired habitual learning.

In the Reversal Trial, mice with more cognitive flexibility were able to recognize the paradigm shift (the platform in the opposite arm of the maze) and quickly escape the maze by swimming to the platform. No baseline differences in cognitive flexibility were detected in prenatally-stressed and non-stressed control (PBS) male mice. However, across PBS and NAC groups, prenatally-stressed mice showed trend enhancements in cognitive flexibility compared to non-stress mice (trend sig., two-way ANOVA testing prenatal stress and NAC treatments: $F[1, 34] = 3.77, p = 0.06$; Fig. 16B). Maternal treatment of AST worsened the cognitive flexibility of male mice overall by increasing their time to find the platform in the Reversal Trial ($F[1, 34] = 5.08, p < 0.05$, Fig 16B). No antioxidant modification of the cognitive flexibility benefit of prenatally-stressed male offspring was found.

In female mice, no baseline differences in habitual learning were detected in prenatally-stressed and non-stressed control (PBS) mice and no main effect of prenatal stress was found (Fig. 16C). A trend interaction of maternal antioxidant treatments with NAC and prenatal stress effects on the habitual learning of female mice was revealed (trend sig., $F[1, 34] = 3.74, p = 0.06$; Fig. 16C). In other words, when NAC was given to mothers prior to prenatal stress, the offspring learned more quickly than offspring of mothers who were given PBS and then prenatally-stressed. This rescue by the NAC antioxidant was shown to be significant when a post-hoc test revealed a decrease in time to the Probe Trial between the prenatally-stressed NAC offspring mice and the prenatally-stressed PBS female mice ($p < 0.01$).
In the Reversal Trial, no baseline differences in cognitive flexibility were detected in prenatally-stressed and non-stressed control (PBS) female mice and no main effect of prenatal stress was found (Fig. 16D). Maternal administration of NAC trend increased the cognitive flexibility of the female mice compared to the control (PBS) mice (trend sig., $F[1, 32] = 3.82, p = 0.06$, Fig. 16D).

**Results for Aim 2.4: Prenatal stress led to increases in PV+-to-GAD67+ cell ratios in male mice, decreases in female mice, and antioxidant treatment eliminated those differences in mFC**

Our lab previously found that prenatal stress caused changes in GABAergic progenitor cell migration into the cerebral cortex and hippocampus (Stevens et al., 2013). In other previous work in our lab, alterations due to prenatal stress on postnatal populations of cortical and hippocampal GABAergic neurons following delays in progenitor migration were investigated. They found that the parvalbumin (PV) subtype of GABAergic interneurons made up a significantly larger proportion of total medial frontal cortical (mFC) GAD67+ cells in prenatally-stressed male offspring (Lussier and Stevens, 2016). Here, the ratio of PV+/GAD67+ cells, the density of PV+ cells, and the density of GAD67+ cells were stereologically assessed in the male and female mFC of adult offspring from either PBS-, NAC-, or AST-treated non-stressed and prenatally-stressed mothers. In males, prenatally-stressed male mice had a higher ratio of PV+/GAD67+ cells in the mFC (two-way ANOVA testing prenatal stress and AST treatments: $F[1, 13] = 9.13, p < 0.01$, Fig. 17A), in accordance with previous findings (Lussier and Stevens, 2016). Interestingly, there was a statistically significant interaction between the effects of prenatal stress and maternal NAC treatment on the proportion of PV+/GAD67+ cells ($F[1, 13] =
When NAC was given to prenatally-stressed mothers, the ratio of PV+/GAD67+ decreased, but when PBS was given to prenatally-stressed mothers, it led to an increase in PV+/GAD67+ cells in the male offspring mFC (Fig. 17A). A post-hoc test revealed a significant decrease in PV+/GAD67+ proportion between the PS PBS control and the PS NAC mice (p < 0.05), demonstrating a correction by maternal NAC of the prenatal stress effect on the proportion of inhibitory neurons that were PV+. Across groups, antioxidant treatments of NAC and AST decreased the ratio of PV+/GAD67+ cells in the mFC compared to PBS (NAC: F [1, 13] = 18.89, p < 0.01 and AST: F [1, 13] = 4.71, p < 0.05), suggesting that maternal antioxidants hindered the maturation of cortical interneurons into this subtype in male offspring.

A closer examination of PV+ and GAD67+ cell densities in the mFC revealed no baseline prenatal stress differences in the densities of GAD67+ cells or PV+ cells in control (PBS) male mFC, unlike the previous findings published by (Lussier and Stevens, 2016). However, maternally-treated AST offspring had trend higher densities of GAD67+ cells in the mFC than the maternally-treated PBS offspring (trend sig., F [1, 14] = 3.92, p = 0.07, Fig. 17B). On the other hand, maternally-treated NAC offspring had lower densities of PV+ cells compared to PBS offspring (trend sig., F [1, 13] = 4.62, p = 0.05, Fig. 17C). This difference in PV+ cortical interneuron subtypes with maternal NAC treatment suggests that the correction of the ratio of PV+/GAD67+ cells with maternal NAC treatment discussed above was driven by NAC impacting PV+ interneuron maturation.

In female offspring mice, a priori t-test found that prenatally-stressed female control (PBS) mice had a lower ratio of PV+/GAD67+ cells in the mFC (p < 0.01, Fig. 17D). Also, prenatal stress led to lower ratios in the PBS and NAC groups of female mice as revealed by a main effect of stress (two-way ANOVA testing for prenatal stress effects and NAC treatment: F
\([1, 10] = 8.46, p < 0.05\). Interactions between the effects of prenatal stress and maternal AST treatments on the proportion of PV+/GAD67+ cells (\(F[1, 12] = 6.40, p < 0.05\)) showed that prenatal stress did not change the proportion of PV+/GAD67+ cells when AST was present.

Further data analysis of the control condition (PBS) for the female mice showed that the difference in the ratios of PV+/GAD67+ cells was entirely driven by a decrease in the PV+ cell density in the mFC and not by increases in the GAD67+ cell density (Fig. 17E-F). A priori t-test found that prenatally-stressed female control (PBS) mice had a lower density of PV+ cells in the mFC \((p < 0.01\), Fig. 17F). Interactions of NAC and AST with prenatal stress effects on PV+ cells were observed (NAC: \(F[1, 10] = 7.25, p < 0.05\), and AST: \(F[1, 13] = 7.30, p < 0.05\)), with prenatal stress resulting in a lower density of PV+ cells in PBS mice, but no differences due to prenatal stress in NAC and AST conditions. While maternal antioxidants prevented any impact of prenatal stress on PV+ cells, resultant densities were not similar to the NS PBS group, and the lack of statistical difference likely was the result of insufficient power in the small sample. An interaction of NAC treatment and prenatal stress was also found for GAD67+ density also (\(F[1, 10] = 6.28\), \(p < 0.05\), Fig. 17E). Because of the small sample, normalization of prenatal stress effect was unclear.

**Results for Aim 2.5: Hippocampal GAD67+ cell densities were reduced by prenatal stress and restored by AST in male mice, and PV+/GAD67+ cell ratio was reduced by prenatal stress and partially restored by NAC in female mice**

PV+/GAD67+ ratios, PV+ cell densities, and GAD67+ cell densities were calculated in the male and female hippocampal CA regions of adult offspring from either PBS-, NAC-, or AST-treated non-stressed and prenatally-stressed mothers. No baseline differences were
detected in prenatally-stressed and non-stressed control (PBS) male offspring PV+/GAD67+ cell proportion and no main effect of prenatal stress was found (Fig. 18A), in contrast to previous results of postnatal GABAergic cell counts in the hippocampal CA (Lussier and Stevens, 2016). Maternal treatments of NAC, but not AST, decreased the PV+-to-GAD67+ cell ratios in the male hippocampal CA (F [1, 10] = 6.28, p < 0.05) similar to its effects in the mFC.

Examining the cell populations contributing to the ratio of PV+/GAD67+ cells, a priori t-test showed that prenatally-stressed male control (PBS) mice had a lower GAD67+ cell density than non-stressed control mice (trend sig., p = 0.07), suggesting there may a primary deficit in hippocampal interneuron populations overall after prenatal stress. Also, prenatal stress led to lower GAD67+ cell densities in male mice across PBS and NAC groups, as revealed by a main effect of stress (two-way ANOVA testing prenatal stress and NAC effects: F [1, 14] = 11.43, p < 0.01, Fig. 18B). An interaction of prenatal stress effects and maternal treatment of AST (F [1, 15] = 5.01, p < 0.05) suggested that AST buffered the effects of prenatal stress on GAD67+ cell densities in the male hippocampal CA. Post-hoc tests revealed a trend increase in GAD67+ cell density in PS AST hippocampus compared to PS PBS hippocampus (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 mice (Fig. 18C).

In female adult offspring mice, a priori t-test found that prenatally-stressed female control (PBS) mice had a lower proportion of PV+/GAD67+ cells in the hippocampal CA (p < 0.01, Fig. 18D), similar to effects of prenatal stress on male offspring previously described (Lussier and Stevens, 2016). Prenatal stress led to lower PV+/GAD67+ cell ratios in female mice as revealed by a main effect of stress (two-way ANOVA testing prenatal stress and AST effects: F [1, 12] = 23.08, p < 0.01, Fig. 18D). An interaction of prenatal stress effects and maternal treatment of
NAC ($F_{[1, 10]} = 8.13, p < 0.05$) revealed that prenatal stress only lowered the ratio of PV+/GAD67+ cells in PBS- or AST-treated mice and not NAC-treated mice. This points to the idea that NAC has the effect of returning the ratio of PV+/GAD67 cells to a balance that is similar to the non-stress, control levels. AST, unlike NAC, did not rescue the effects of prenatal stress and prenatally-stressed AST mice had significantly lower PV+/GAD67+ ratios compared to non-stressed AST mice (post-hoc, $p < 0.05$).

A closer examination of proportions of PV+ and GAD67+ cell densities independently in the hippocampal CA revealed no baseline differences in the densities of GAD67+ cells in control (PBS) female mice (Fig. 18E). This was in contrast to the male mice, wherein the PV+/GAD67+ ratio differences were mainly driven by the differences in GAD67+ cell densities (Fig. 18A-B). In the female mice, however, differences in proportion were driven by impacts on the cell densities of the PV subtype (Fig. 18F). A priori t-test found that prenatally-stressed female PBS mice had a trend lower proportion of PV+ cells in the hippocampal CA (trend sig., $p = 0.06$, Fig. 18F), suggesting that prenatal stress may have impaired PV+ cell maturation in females. AST-treated offspring mice had PV+ cell densities similar to the non-stressed and prenatally-stressed PBS mice, revealing a main effect of prenatal stress ($F_{[1, 12]} = 14.77, p < 0.01$, Fig. 18F). AST did not rescue the effects of prenatal stress and prenatally-stressed AST mice had significantly lower densities of PV+ cells compared to non-stressed AST mice (post-hoc, $p < 0.05$).

**Results for Aim 2.6: GAD67+ cell densities across regions correlated significantly with anxiety-like behavior in both male and female mice and social behavior in female mice**

Similar to previous results (Lussier and Stevens, 2016), correlations were uncovered between GAD67+ cell densities in the mFC and hippocampal CA and behavior of individual
animals. The GAD67+ cell densities in the mFC correlated significantly with the amount of time the female mice spent in the center of the OFT during the first 5 minutes of the test ($R = -0.67, p < 0.01$, Fig. 19A) and trended significantly with the amount of time spent in the closed arm of the EPM ($R = 0.41, p = 0.06$, Fig. 19B). In the hippocampal CA, GAD67+ cell densities correlated with the ratio of time spent in proximity to the novel mouse cup to the familiar mouse cup in female mice ($R = 0.46, p < 0.05$, Fig. 19C). In male mice, hippocampal CA GAD67+ cell densities and the amount of time the mice spent in the center of the OFT during the first 5 minutes of the test also trend correlated ($R = 0.36, p = 0.07$, Fig. 19D).

**Aim 2 conclusions and discussion**

Maternal stress during pregnancy is known to cause a range of inhibitory or anxiety-like behaviors in rodent offspring which include reduced social recognition (Harmon et al., 2009), increased anxiety-like behavior in the light-dark box and the elevated plus maze (Laloux et al., 2012; Grigoryan and Segal, 2013), and reduced activity in the center of the open field (Laloux et al., 2012; Grigoryan and Segal, 2013). Prenatal stress also affects adult CD1 mouse offspring behavioral outcomes and that altered behavior correlates with neurobiological differences in individual mice (Lussier and Stevens, 2016). My goal in this Aim was to test the hypothesis that prenatal stress-mediated redox dysregulation in the embryonic brain during development of GABAergic systems would have lasting effects on the adult offspring behavioral outcomes.

In this study, I found that prenatal stress increased anxiety-like behavior in both sexes but, while astaxanthin (AST) and N-acetylcysteine (NAC) prevented the anxiety-like phenotype in male mice, only AST prevented anxiety-like behavior in female mice. Impairments in sociability was caused by prenatal stress in female mice, but was not prevented by either
maternal antioxidant treatment. Prenatal stress had no effect on preference for social novelty in either sex. Deficits in sensorimotor gating were observed in prenatally-stressed male mice and deficits were prevented by either NAC or AST treatment. For female offspring mice, maternal antioxidant treatments of NAC and AST alone (without the effects of prenatal stress) improved the sensorimotor gating of the startle reflex. Prenatally-stressed resulted in no difference in activity levels of the male or female offspring mice. Both prenatally-stressed male and female mice exhibited retarded motor learning on the rotarod and neither antioxidant treatment prevented arrested motor learning in prenatally-stressed offspring mice. Prenatally-stressed male mice showed more cognitive flexibility on the water T-maze with AST worsening cognitive flexibility. Prenatally-stressed females, on the other hand, showed improvements in habitual learning and cognitive flexibility with maternal NAC treatments. Prenatal stress led to increases in PV+/GAD67+ cell ratios in mFC in male mice, but decreases in female mice, and antioxidant treatments eliminated those differences. Hippocampal GAD67+ cell densities were reduced by prenatal stress and restored by AST in male mice, and PV+/GAD67+ cell ratio was reduced by prenatal stress and partially restored by NAC in female mice. Lastly, GAD67+ cell densities across regions correlated significantly with anxiety-like behavior in both male and female mice and social behavior in female mice. These results suggest that prenatal stress-mediated redox dysregulation in the embryonic brain during development has lasting effects on some adult offspring behavioral outcomes and that by altering the redox balance through maternal administration of antioxidants at the time of insult, some behavioral phenotypes can be prevented.

The effects of maternal prenatal stress on increasing anxiety-like behavior in offspring rodents has been documented in several studies (Vallée et al., 1997; van den Hove et al., 2005;
Pallarés et al., 2007; Miyagawa et al., 2011; Lussier and Stevens, 2016). In this study, the elevated plus maze (EPM) revealed an increased anxiety-like phenotype in the prenatally-stressed male and female mice who preferred to stay in the closed arms of the maze as opposed to the open arms of the maze (Fig. 10B and 10E), as measured by somewhat differing patterns of results for males and females. AST, but not NAC, partially prevented the anxious-like phenotype in male and female mice, suggesting that antioxidant treatment may buffer the redox imbalance generated by maternal prenatal stress. In fact, redox imbalance during embryonic development is known to influence offspring behavior. In one study, pregnant mice were exposed to lipopolysaccharide (LPS) with the expressed purpose of altering the redox signaling and cellular levels of reactive oxygen species (ROS) in the embryonic brain by inducing maternal immune activation (MIA) (Le Belle et al., 2014). Pups from MIA mothers displayed autism-associated and anxiety-like behaviors, including deficits in ultrasonic vocalizations, excessive repetitive grooming behavior, lower social interaction in the social chamber, and decreased time in the open arms of the EPM. MIA stimulates the generation of ROS through the induction of several cytokines and activation of NADPH oxidase (NOX) (Weigent and Blalock, 1997; Le Belle et al., 2014) and the researchers decided to test the ability of NOX inhibition to rescue the two most significantly abnormal behavior caused by MIA: vocalization and grooming. They found that in vivo inhibition of the ROS-generating enzyme NOX following LPS treatment rescued the excessive grooming behavior, but not the early vocalization deficit (Le Belle et al., 2014). Our study found that maternal treatments of AST, but not NAC, prevented prenatally-stressed induced anxiety-like behaviors in male and female offspring mice (Fig. 10B and 10E) on the EPM task.
The EPM task was not the only measure of anxiety-like behavior we used and the open field test (OFT) revealed somewhat different results. Unlike previous findings (Lussier and Stevens, 2016), we did not find that prenatal stress caused anxiety-like behavior in either male or female adult offspring mice. The difference in results could lie in the testing parameters and data analysis: we chose to test for anxiety-like behavior during the first 5 minutes on Day 1 of the OFT and Lussier and Stevens (2016) found a difference during the full 30 minutes on Day 2. While our results on the OFT did not reveal an increased anxious-like phenotype in either the male or female prenatally-stressed mice, NAC and AST independently increased the amount of time male mice spent in the center of the open-field arena during the first 5 minutes of the task (Fig. 9A), interpreted to be a reduced anxiety-like phenotype. This prevention of anxiety-like behavior on the OFT may reflect a sex-specific effect of NAC and AST, wherein maternal antioxidant administration differentially affected male and female embryos at the time of prenatal stress. The manifestations of sexually dimorphic impairments in behavioral phenotypes caused by prenatal stress in male and female offspring mice has been observed in other studies (Bowman et al., 2004). Exposure to stress during gestation results in alterations to several aspects of brain development, interfering with the expression of normal behavior, neuroendocrine, and neurochemical responses in a sex-specific manner. Bowman et al. (2004) found that female mice were less anxious than male rats on the OFT and these behavioral changes were associated with a decrease in dopamine activity in the prefrontal cortex (PFC) of male rats and an increase in dopamine activity in the PFC in female rats. In other words, maternal prenatal stress modifies neuroendocrine, cognitive, and neurochemical profiles in adult offspring in a sex-specific manner.
Social behavior is critically affected by prenatal maternal stress, typically resulting in reduced social abilities in offspring (Lussier and Stevens, 2016). By spending less time with the mouse cup than their non-stressed counterparts (Fig. 11E), prenatally-stressed control female mice exhibited a decreased sociability preference. Similar to the increased anxiety-like phenotype in females, this inhibited sociability was partially rescued by AST. This was apparent from a significant decrease in the time spent with the empty cup and increased time spent with the mouse cup in the three chamber sociability task (Fig. 11D-E). Prenatal stress had no effect on preference for social novelty in either sex offspring (Fig. 12), similar to what was previously found by Lussier and Stevens (2016), suggesting that prenatal stress does not impair social recognition in offspring. Surprisingly, prenatally-stressed male mice did not show deficits in sociability as previously found (Lussier and Stevens, 2016). Moreover, the control mice (PBS) did not show a preference greater than chance (preference > 0.5 ratio) for ratio of time spent with mouse cup:empty cup, suggesting no preference for either cup regardless of prenatal stress and antioxidant treatment. One of the defining symptoms of autism is inhibited sociability and the prevalence of autism is higher in males than in females (Richer, 1978; Davis and Pfaff, 2014). The female-specific findings in this study highlights the difficult nature of measuring a complex behavior like sociability and social preference in rodents which, like all behavioral tasks, is limited in its analogy to human phenomena.

In the sensorimotor domain, several behavioral assays were conducted to examine: sensorimotor gating with prepulse inhibition (PPI), locomotor activity on Day 2 of the OFT, motor learning on the rotarod, and habitual learning and cognitive flexibility on the water T-maze. Prenatally-stressed control (PBS) male mice displayed deficits in sensorimotor gating on the PPI test across all 3 dB levels (Fig. 13C). Similar to male mice, prenatally-stressed control
(PBS) female mice displayed deficits in sensorimotor gating, but only at the 5 dB level, with no differences in gating their startle reflex at higher dB levels (Fig. 13D). This deficit is similar to previous findings in prenatally-stressed adult offspring mice (Matrisciano et al., 2013), and is seen clinically in patients with schizophrenia (for a review, please see Braff et al. (2001)). Both NAC and AST treatments prevented the sensorimotor deficits in male mice and improved the sensorimotor gating of the startle reflex in female mice (without the effects of prenatal stress). This suggests that redox imbalance in the embryonic brain caused by prenatal stress can be alleviated by maternal antioxidant administration and can prevent the sensorimotor gating deficits observed in male offspring mice. Although both sexes demonstrated deficits in sensorimotor gating, males exhibited higher baseline startle amplitudes than female mice. This is consistent with previous research examining the effects of sex on modulation of the acoustic startle response in C57BL/6J and C3H mice (Plappert et al., 2005).

Time-in-zones on Day 1 of the OFT was used to probe for anxiety-like behaviors. On Day 2 of the OFT, when responses to novelty are diminished, distance traveled was used as a measurement of locomotor activity. Generally, hyperactivity is characteristic of prenatally-stressed offspring on the OFT (Peters, 1986; Matrisciano et al., 2013), but other researchers have found that prenatally-stressed rats exhibit hyperactivity in the first 5 minutes of the OFT, but then their activity returns to levels comparable to the control rats (Vallée et al., 1997). In this study, prenatal stress resulted in no difference in activity levels of either the male or female offspring mice over the course of the 30 minute trial (Figs. 14A-B). One major difference between the findings of past research and our findings here is that our locomotor measure is less tied to anxiety-like behaviors because we measured distance traveled on Day 2. Our mice were already familiar with the open field arena and their locomotor behavior was a more pure measure
of activity rather than anxiety or exploration of a novel environment. In male offspring mice, AST resulted in trend hyperactivity, but only in prenatally-stressed mice (trend sig., Fig. 14A), and in female offspring, NAC caused hyperactivity, but only in non-stressed mice (Fig. 14B). These contradictory results point to the fact that our maternal antioxidant treatments did not have the same effects on locomotion and, in fact, our antioxidants had different effects on the sexes.

Prenatally-stressed male and female mice showed impairments in motor learning as evidence by lower learning coefficients on the rotarod task (Fig. 15). Neither maternal antioxidant treatments rescued the motor learning of either male or female offspring. From this result and the results from Aim 1, we can posit that restoring redox balance in the embryonic brain does not prevent prenatally-stressed-induced impairments to motor learning in offspring mice.

Habitual learning and cognitive flexibility were assessed using two different phases of testing in the water T-maze. Prenatal stress caused varied changes in habitual learning and cognitive flexibility deficits (perseverative behaviors) in male and female offspring (Fig. 16). In habit learning assessment, prenatally-stressed control (PBS) males and female mice were no worse than their respective controls (Fig. 16A, C). Deficits in performance on the Reversal Trial were in opposite directions in male depending on maternal antioxidant treatment—NAC males had less perseverative behavior and better cognitive flexibility while AST males showed perseverative behavior and less cognitive flexibility, as implicated in schizophrenia, autism, obsessive compulsive disorders, and addictive behavior (Ridley, 1994). NAC improved the habitual learning in female mice and prevented the perseverative behaviors observed in the adult offspring female mice. This is yet another instance in which the two different maternal antioxidant treatments had divergent results on the behavioral outcome.
The behavioral results outlined above underscore the significance of the prenatal stress model in understanding neuropsychiatric disorders. By also examining in these same adult mice the association of these behaviors with GABAergic cell populations in the forebrain, we may begin to provide a possible mechanism for how exposure to prenatal stress during gestation can be a risk factor for psychiatric disorders in adulthood (Lussier and Stevens, 2016). Examining the total GABAergic cell population and the maturation of a significant minority into Parvalbumin-expressing neurons (PV+) in offspring neocortex after prenatal stress and maternal antioxidants was the goal.

In typical mouse forebrain development, GABAergic neuron cell populations increase in early postnatal period until reaching an apex and then undergoing pruning by programmed cell death (Southwell et al., 2012). However, according to the proposed model postulated by Lussier and Stevens (2016), prenatal stress delays the cellular processes that bring about populating, pruning, and differentiation (Patz et al., 2003; Southwell et al., 2012). By adulthood (approximately P150 in previous work), populations of GABAergic cells normalized between non-stress and prenatally-stressed offspring.

In order to examine whether these abnormalities may be due to redox imbalance in embryonic brain development, GAD67+ cell mFC and hippocampal densities were measured. Findings were generally compatible with what has been previously reported by Lussier and Stevens (2016), including the normal GAD67+ total population in mFC and normal PV+ total population in the hippocampus. A trend decrease in male hippocampal GAD67+ cell density (Fig. 18B) in our findings here suggests a primary deficit in hippocampal interneuron populations overall after prenatal stress that was not previously detected. I also observed no differences in the ratio of PV+/GAD67+ in the hippocampal CA and no differences in the ratio
of PV+ cell density, in contrast to previous findings (Lussier and Stevens, 2016). The efficacy of shifting the redox balance in the embryonic brain during gestation for normalizing cell populations after prenatal stress was unclear from these data, as the effects of prenatal stress had divergent results on GAD67+ cell populations in the male hippocampal CA. On the one hand, AST prevented the reduction in GAD67+ cells and restored them to levels comparable to the control, but on the other hand, NAC had no effect and they remained significantly lower than the NS NAC comparison (Fig. 18B). These findings once again point to the differential effects of NAC and AST on postnatal GABAergic cell populations.

In female mouse cortical interneuron populations after prenatal stress, which this study is the first to examine, PV+ neurons made up a significantly smaller proportion of total mFC and hippocampal CA GAD67+ cells in prenatally-stressed female offspring. The lower levels of PV+ cells in both regions, as observed here, is also observed in the pathophysiology of schizophrenic patients. Often called the “loss of GABA phenotype” in the literature (Lewis et al., 2012), the decrease in GAD67+ cells occurs disproportionally in PV interneurons, and may be characteristic of schizophrenia (Beasley and Reynolds, 1997; Hashimoto et al., 2003). PV+ interneurons in the developing cortex may be particularly vulnerable to redox dysregulation because of their prolonged period of maturation (Behrens and Sejnowski, 2009). In mice, other rodents, and even primates, maturation of the PV+ interneurons occurs postnatally (Huang, 2009), but the progenitor cells complete their migration from the ganglionic eminences at embryonic day 15 (E15) (Wonders and Anderson, 2006). Although the undifferentiated cells reach the neocortex at E15, they remain silent until the beginning of the second postnatal week after which time they begin expressing PV (Cristo et al., 2004). It is possible that redox
dysregulation during their migration could initiate the processes that eventually lead to the loss of GABAergic phenotypes observed in neuropsychiatric disorders.

In order to target the late prenatal/early postnatal developmental period, studies have used the DISC1 model, the maternal-immune activation model, and the prenatal exposure to methylazoxymethanol acetate (MAM) to demonstrate that normal brain development can be derailed during these specific developments periods by perturbations that lead to decreased number of PV+ interneurons (Meyer et al., 2006; Hikida et al., 2007; Lodge and Grace, 2007; Lodge et al., 2009). In one particular study, mitotoxin was given intraperitoneally to pregnant rats at E17, which coincides with interneuron proliferation and migration into the neocortex (Lodge and Grace, 2007), and they observed a decrease in PV+ interneurons in the hippocampus and other brain regions in adulthood. In another study, increases in levels of pro-inflammatory cytokines, interleukin-6 (IL-6) which is known to induce the superoxide-producing NADPH oxidase (Nox2) pathway, led to the loss of GABAergic phenotype in PV+ interneurons and caused a depression of inhibitory activity in the prefrontal cortex (for a review, please see Behrens and Sejnowski (2009). Our results suggest that, at least in female mice, prenatal stress induced redox dysregulation in the migrating GABAergic progenitors in the offspring brain and that the maturation of PV+ interneurons in the mFC and the hippocampus was affected by the imbalance of redox processes. Our hypothesis is supported by findings that antioxidants partially prevented the low levels of PV+ cells in the mFC (Fig. 17F) and the hippocampal CA (Fig. 18F).

The behavioral results and cortical interneuron population densities and proportions reviewed above highlight the relevance of the prenatal stress model for neuropsychiatric disorders. Prenatally-stressed female offspring mice exhibited increased anxious-like behavior, as shown through the EPM and the OFT (Fig. 9B, 10D-F). Across mice in all six groups used in
the study, these behaviors correlated with mFC and hippocampal GAD67+ cell density. Specifically, higher densities of GAD67+ cells in the mFC was correlated with a less anxious phenotype in female mice, as seen by significant correlations between GAD67+ cells and more time in the center of the arena in the OFT (Fig. 19A) and less time in the closed arm of the EPM (Fig. 19B). Although no sociability or social recognition differences were observed between prenatally-stressed and non-stressed female mice, more GAD67+ cells in the hippocampal CA correlated with greater social recognition of the familiar mouse (Fig. 19C). These correlations between GABAergic cell populations and behavior were found to be independent of stress and/or antioxidant group effects. These correlations are particularly important because of the links between abnormal inhibitory cortical neuron functioning and the psychopathology of anxiety disorders and autism, a disorder characterized by impairments in sociability (for a comprehensive review see Fine et al. (2014)).

Male mice also exhibited a less anxious-like phenotype on the OFT that positively correlated with GAD67+ cell populations in the hippocampus (Fig. 19D). Because antioxidants were shown to have a significant influence on the amount of time male mice spent in the center of the OFT during the first 5 minutes (Fig. 9A) as well as on the density of GAD67+ cells in the hippocampus, the correlation might not be completely independent of these group stress and antioxidant effects. Moreover, this correlation indicates that maternal antioxidant treatment (NAC and AST) during prenatal stress increased the GAD67+ cell population in the hippocampus and this was associated with a less anxious-like phenotype in male offspring mice. These correlations also suggest that prenatal stress has sexually dimorphic effects on cortical interneuron populations and the manifestations of anxious-like phenotypes in male and female offspring mice (Davis and Pfaff, 2014).
To our knowledge, these experiments are the first to investigate whether redox
dysregulation during pregnancy mediates the effect prenatal stress has on anxiety-like behaviors,
social behaviors, sensorimotor behaviors, and postnatal GABAergic neuron populations in the
brains of adult offspring mice. Several studies have shown that redox dysregulation may be a
component of physiological stressors that occur during pregnancy (Peuchant et al., 2004; Cambonie et al., 2007b; Derks et al., 2010a; Ziech et al., 2011) and that GABAergic interneurons
are vulnerable to redox dysregulation, even during late prenatal/early postnatal periods (Meyer et al., 2006; Hikida et al., 2007; Lodge and Grace, 2007; Behrens and Sejnowski, 2009; Lodge et al., 2009). The data from Aim 1 demonstrated that maternal treatments of antioxidants may
equilibrare the prenatal stress-induced redox dysregulation of the embryonic brain during
gestation and may buffer some of the negative, long-lasting consequences of prenatal stress on
the embryonic brain. That buffer may prevent changes in some behavioral domains—as shown
here, those offspring behavioral changes with prenatal stress sensitive to maternal antioxidants
were anxiety-like behavior, sociability, and sensorimotor deficits. On the whole, neither NAC
nor AST was more effective than the other in preventing prenatal stress-induced aberrant
behavior in male or female offspring mice. For anxiety-like behavior revealed by the EPM task,
AST prevented anxious-like phenotypes in males and females. On the OFT, NAC and AST
alone (without the effects of prenatal stress) produced a less anxious-like phenotype in males
only. For deficits induced by prenatal stress on the PPI test, NAC and AST prevented deficits in
male mice and NAC and AST alone (without the effects of prenatal stress) enhanced the
sensorimotor gating in female mice. On the water T-maze, NAC alone (without the effects of
prenatal stress) improved habitual learning and cognitive flexibility in female mice. As for
postnatal GABAergic cell populations, AST prevented the reduction in GAD67+ cells in the
hippocampal CA in male mice and NAC and AST partially prevented the reduction in PV+ cells in the mFC. Neither NAC nor AST revealed itself to be a more effective antioxidant in preventing postnatal GABAergic cell population differences in the mFC or the hippocampus in either male or female offspring mice.

These results help to elucidate poorly understood mechanisms of risk factors for mental illness and indicate that targeting an appropriate balance of redox biology during neurodevelopment may have benefits for offspring brain and overall development.
(A) No prenatal stress baseline differences or main effects of stress in male offspring were found.

NAC and AST significantly increased the amount of time male mice spent in the center of the OFT.

Figure 9. NAC and AST increased the amount of time PS male mice spent in the center of the OFT.

(A) No prenatal stress baseline differences or main effects of stress in male offspring were found. NAC and AST significantly increased the amount of time spent in the center of the arena.
Figure 9 – continued. (## $p < 0.01$ and $\# p < 0.05$, respectively by two-way ANOVA and * $p < 0.05$ compared to PS PBS). (B) No prenatal stress baseline differences or main effects of stress in female offspring were found.
Figure 10. PS caused male and female mice to spend less time in the open arms of the EPM and AST partially prevented the anxiety-like phenotype.

(A) PS PBS male mice spent more time in the closed arm, suggesting an anxious-like phenotype ($p < 0.05$). Prenatally-stressed male mice spent more time in the closed arm of the EPM.
**Figure 10 – continued.**  
(α < 0.05 by two-way ANOVA comparing PBS and NAC mice) and an interaction of AST and prenatal stress demonstrated that AST did not rescued anxiety-like behavior (ϕ < 0.05 by two-way ANOVA).  
(B) A main effect of stress was found in the ratio of time spent in the open arm to the closed arm of the EPM (α < 0.05 by two-way ANOVA comparing PBS and NAC mice).  
(C) A main effect of stress was found in the ratio of open:closed arm entries in male mice (α < 0.05 by two-way ANOVA comparing PBS and NAC mice).  
(D) No prenatal stress baseline differences or main effects of stress in female offspring of time spent in the closed arm of the maze were revealed.  
(E) Prenatal-stressed females spent significantly less time in the open arms of the maze, suggesting an anxious-like phenotype (α < 0.05 by two-way ANOVA comparing PBS and AST mice).  
Post-hoc tests revealed that NS AST female mice spent significantly more time in the open:closed arm of the EPM compared to PS AST mice (ϕ < 0.05), suggesting an impact on offspring from AST alone.  
(F) A priori t-test found that PS PBS female mice had fewer open:closed arm entries, suggesting an anxious-like phenotype (ϕ < 0.05).  
Prenatally-stressed mice had fewer open:closed arm entries compared to non-stressed female mice (NAC: αα < 0.01 and AST: α < 0.05 by two-way ANOVAs compared to the PBS group).  
AST treatment, but not NAC treatment, increased the ratio of open:closed arm entries (ϕ < 0.05), signifying that AST ameliorated the anxious-like phenotype observed in the prenatally-stressed female mice.
Figure 11. PS impaired sociability in female mice and was not prevented by either antioxidant.

(A) Prenatally-stressed male mice spent less time with the empty cup compared to non-stress mice ($\alpha p < 0.05$ by two-way ANOVA comparing PBS to NAC mice). (B) No differences in
Figure 11 – continued.  time spent in proximity to the mouse cup or (C) the ratio of time spent with the mouse cup to time spent with the empty cup. (D) Interactions with NAC and AST show that concurrent antioxidant administration and prenatal stress (PS AST) return female mice sociability to levels comparable to NS PBS ($\phi p < 0.05$ by 2, two-way ANOVAs and post-hoc t-test $p < 0.05$ [AST], compared to PS PBS). (E) Prenatally-stressed female mice spent less time with the mouse cup, indicating a deficit in sociability (trend, $p = 0.05$) with PS AST spending less time with the mouse cup than NS AST mice (post-hoc, & $p < 0.05$). (F) No differences were found in the ratio of time spent with the mouse cup to the empty cup across the groups.
Figure 12. PS did not affect male or female social preference for a novel mouse.

No prenatal stress baseline differences or main effects of stress in male offspring behavior were found for time spent with familiar mouse (A), time spent with the novel mouse (B), or time with novel:familiar mouse (C). (D) While no prenatal stress baseline differences or main effects of
**Figure 12 – continued.** Stress in female offspring were observed in time spent with the familiar mouse, maternally-treated AST female mice spent less time with the familiar mice (# $p < 0.05$ by two-way ANOVA). No differences in time spent with the novel mouse (E) or the ratio of time spent with novel:familiar (F) were detected in the female offspring mice.
Figure 13. PS caused sensorimotor deficits in male mice; NAC and AST prevented the deficits in male mice and improved sensorimotor gating in female mice.
**Figure 13 – continued.** (A) No differences in the baseline startle response was observed between male offspring groups. (B) No differences in the baseline startle response was observed between female offspring groups. (C) PS PBS male mice showed deficits in PPI across all three decibel (dB) levels (α $p < 0.05$ by two-way ANOVA). NS AST male mice showed deficits in 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 improvements in sensorimotor gating compared to PS PBS male mice (post-hoc, trend sig., $p = 0.07$). (D) An interaction between NAC and prenatal stress was found and suggests that NAC on its own is causing female mice to perform poorly on the PPI (ϕ $p < 0.05$ 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, * $p < 0.05$ and * $p < 0.05$, respectively). At the 10 dB level, only PS NAC showed improvements in sensorimotor gating compared to PS PBS female mice (post-hoc, * $p < 0.05$).
Figure 14. PS did not cause changes in locomotion in male and female mice.

(A) A trend interaction of AST and prenatal stress effects was found (trend sig., $\phi p = 0.06$ by two-way ANOVA), suggesting AST may affect locomotor activity differently than PBS, depending on whether the mouse experienced prenatal stress. (B) No baseline differences were detected in PS PBS and NS PBS female mice and no main effects of prenatal stress were found.
Figure 14 – continued. 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 ($p < 0.05$ by post-hoc t-test) and NS PBS ($p < 0.05$ by post-hoc t-test).
Figure 15. PS male and female mice exhibited retarded motor learning on the rotarod and neither NAC nor AST prevented arrested motor learning.
**Figure 15 – continued.** (A-C) Prenatally-stressed male mice had a lower learning ratio (learning coefficient) than non-stressed mice ($\alpha\alpha \ p < 0.01$ by two separate, three-way ANOVAs for NAC and AST). All three treatment groups showed daily learning. Interactions of NAC and AST with prenatal stress effects were found ($\phi \ p < 0.05$ and $\phi\phi \ p < 0.01$, respectively by two-way ANOVA). A main effect of AST treatment suggests an enhancement in motor learning for the non-stressed male mice ($# \ p < 0.05$ by three-way ANOVA). (D-F) Prenatally-stressed female mice had a lower motor learning ratio (learning coefficient) than non-stressed mice ($\alpha\alpha \ p < 0.01$ by three-way ANOVA testing for NAC treatment effects). All three treatment groups showed daily learning. Interactions of NAC with prenatal stress effects were found ($\phi\phi \ p < 0.01$ by three-way ANOVA). A main effect of AST treatment suggests an enhancement in motor learning for the female mice ($# \ p < 0.05$ by three-way ANOVA).
Prenatally-stressed male mice showed deficits in habitual learning compared to non-stressed mice (αα \( p < 0.01 \) by two-way ANOVA). A significant interaction was detected suggesting that prenatal stress and AST exacerbated habitual learning (ϕϕ \( p < 0.01 \) by two-way ANOVA). Post-hoc tests revealed that PS AST male mice showed impaired habitual learning compared to PS PBS (** \( p < 0.01 \) by post-hoc t-test) and NS AST (&& \( p < 0.01 \) by post-hoc t-test). (B)

Figure 16. PS male mice showed more cognitive flexibility on WTM with AST worsening cognitive flexibility; PS females showed improvements in habitual learning and cognitive flexibility with maternal antioxidants.

(A) Prenatally-stressed male mice showed deficits in habitual learning compared to non-stressed mice (αα \( p < 0.01 \) by two-way ANOVA). A significant interaction was detected suggesting that prenatal stress and AST exacerbated habitual learning (ϕϕ \( p < 0.01 \) by two-way ANOVA). Post-hoc tests revealed that PS AST male mice showed impaired habitual learning compared to PS PBS (** \( p < 0.01 \) by post-hoc t-test) and NS AST (&& \( p < 0.01 \) by post-hoc t-test). (B)
Figure 16 – continued. Prenatally-stressed male mice showed enhancements in their cognitive flexibility compared to non-stressed mice (trend sig., \( p = 0.06 \) by two-way ANOVA testing prenatal stress and NAC treatment effects). AST mice demonstrated impaired cognitive flexibility during the Reversal Task (# \( p < 0.05 \) by two-way ANOVA). (C) Trend interactions were found between NAC and prenatal stress effects (\( \phi p = 0.06 \) by two-way ANOVA). The rescue of NAC was revealed to be statistically significant between PS NAC and PS PBS (** \( p < 0.01 \) by post-hoc t-test). (D) A trend NAC treatment effect improved cognitive flexibility in the female mice compared to the PBS mice (trend, # \( p = 0.06 \) by two-way ANOVA).
Figure 17. PS led to increases in PV+/GAD67+ cell ratios in mFC in male mice, but decreases in female mice, and antioxidant treatments prevented those differences.
Figure 17 – continued. (A) Prenatally-stressed male mice had a higher ratio of PV+/GAD67+ cells in the mFC ($α α p < 0.01$ by two-way ANOVA testing prenatal stress effects and AST treatment). A prenatal stress by NAC interaction was found in PV+/GAD67+ cells ($ϕ p < 0.05$ 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) AST-treated offspring had trend higher densities of GAD67+ cells in the mFC than the maternally-treated PBS offspring (trend sig., $p = 0.07$ by two-way ANOVA). (C) NAC-treated offspring had lower densities of PV+ cells compared to PBS offspring (trend sig., $p = 0.05$ by two-way ANOVA). (D) A priori t-test found that PS PBS female mice had a lower ratio of PV+/GAD67+ cells in the mFC ($$$ p < 0.01$ by t-test). Prenatal stress led to lower ratios of PV+/GAD67+ cells in female mice as revealed by a main effect of stress ($α p < 0.05$ by two-way ANOVA testing prenatal stress effects and NAC treatment effects). An interaction between the effects of prenatal stress and maternal AST treatments on the proportion of PV+/GAD67+ cells ($ϕ p < 0.05$) suggests that AST only partially restored the proportion of PV+/GAD67+ cells to the levels of the NS PBS. (E) An interaction of NAC treatment and prenatal stress was found for GAD67+ density ($ϕ p < 0.05$ by two-way ANOVA). (F) A priori t-test found that PS PBS female mice had a lower density of PV+ cells in the mFC ($$$ p < 0.01$ by t-test). Interactions of NAC and AST with prenatal stress effects on PV+ cells were observed ($ϕ p < 0.05$ and $ϕ p < 0.05$, respectively), but the small sample size prevents any conclusions about antioxidant rescue effects from being made.
Figure 18. Hippocampal GAD67+ cell densities were reduced by PS and restored by AST in male mice, and PV+/GAD67+ cell ratio was reduced by PS and partially restored by NAC in female mice.
Figure 18 – continued.  (A) No baseline differences were detected in PS PBS and 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 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 testing prenatal stress and NAC treatment effects). Post-hoc tests found that PS NAC mice had lower GAD67+ cell densities than NS NAC mice (& $p < 0.05$). An interaction of prenatal stress effects and maternal treatment of AST ($ϕ p < 0.05$ by two-way ANOVA) revealed that prenatal stress only lowered GAD67+ in PBS-treated mice and not AST-treated mice. Post-hoc tests revealed a trend increase in GAD67+ cell density in PS AST hippocampus compared to PS PBS hippocampus (trend sig., $p = 0.07$). (C) No differences in PV+ cell densities were detected in the hippocampal CA between any of the groups of male offspring mice. (D) A priori t-test found that PS PBS mice 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 ($ϕ p < 0.05$ by two-way ANOVA) revealed that prenatal stress only lowered the ratio of PV+/GAD67+ cells in PBS-treated mice and not NAC-treated mice. AST did not rescue the effects of prenatal stress and PS AST mice had significantly lower PV+/GAD67+ ratios compared to NS AST mice (& $p < 0.05$). (E) No baseline differences in the densities of GAD67+ cells in control (PBS) female mice and no main effects of stress were found. (F) A priori t-test found that PS PBS female mice had a trend lower proportion of PV+
Figure 18 – continued. 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 ($\alpha p < 0.01$ 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 ($& p < 0.05$).
Correlations with Behavior

(A) GAD67+ cell densities in the mFC correlated significantly with the amount of time the female mice spent in the center of the OFT during the first 5 minutes of the test (R² = 0.45, p < 0.01 by Pearson correlation) and (B) trended significantly with the amount of time spent in the closed arm of the EPM (trend, R² = 0.17, p = 0.06 by Pearson correlation). (C) In the hippocampal CA, GAD67+ cell densities correlated with the ratio of time spent in proximity to the novel mouse cup to the familiar mouse cup in female mice (R² = 0.21, p < 0.05 by Pearson correlation). (D) In male mice, hippocampal CA GAD67+ cell densities and the amount of time

Figure 19. GAD67+ cell densities across regions correlated significantly with anxiety-like behavior in both male and female mice and social behavior in female mice.

(A) GAD67+ cell densities in the mFC correlated significantly with the amount of time the female mice spent in the center of the OFT during the first 5 minutes of the test (R² = 0.45, p < 0.01 by Pearson correlation) and (B) trended significantly with the amount of time spent in the closed arm of the EPM (trend, R² = 0.17, p = 0.06 by Pearson correlation). (C) In the hippocampal CA, GAD67+ cell densities correlated with the ratio of time spent in proximity to the novel mouse cup to the familiar mouse cup in female mice (R² = 0.21, p < 0.05 by Pearson correlation). (D) In male mice, hippocampal CA GAD67+ cell densities and the amount of time
Figure 19 – continued. the mice spent in the center of the OFT during the first 5 minutes of the test also correlated ($R^2 = 0.13, p = 0.07$ by Pearson correlation).
CHAPTER 5. CONCLUSIONS

Maternal stress during gestation is associated with an increased risk for neuropsychiatric disorders in her offspring, such as schizophrenia (King and Laplante, 2005; Bale, 2009) and autism spectrum disorder (Ward, 1990; Beversdorf et al., 2005; Ronald et al., 2011). Stress alters multiple aspects of physiology (McEwen, 2004; Sousa and Almeida, 2012) that, when occurring in a pregnant mother, may pass to the fetus and alter the developing brain. There are important relationships between the GABAergic neural circuitry of the forebrain and mental illness (Benes and Berretta, 2001; Hashimoto et al., 2008; Yip et al., 2008). Perturbations of inhibitory forebrain systems during embryonic development can have significant, enduring consequences for neural function in adolescence and adulthood (Powell et al., 2003; Levitt et al., 2004; Fu et al., 2012). Inhibitory cortical interneurons are of particular significance because of their role in cognition (Fishell and Rudy, 2011a) and links between atypical GABAergic system functioning and the psychopathology of schizophrenia, autism, and anxiety disorders (for a comprehensive review see Fine et al. (2014)).

The overall goal of this project was to examine the role that redox dysregulation plays in the effects of prenatal stress on the developing brain. The purpose of Aim 1 was to examine the effects of redox dysregulation on the migration of GABAergic progenitor cells in the embryonic brain to test whether maternal treatments with antioxidants before prenatal stress ameliorate the deleterious effects caused by prenatal stress in the embryonic brain. Our results suggest that redox dysregulation is one of the mediators in the effect of prenatal stress has on the migration patterns of interneuron progenitors. We found that both maternal antioxidant treatments, N-acetylcysteine (NAC) and astaxanthin (AST), rescued delays in cortical interneuron migration
due to prenatal stress. We chose to two different antioxidant treatments to measure the effects of each antioxidant treatment individually while also looking for common trends and convergent results between the two treatments. In both male and female offspring, a prevention of prenatal stress-induced anxiety-like behavior on the elevated plus maze (EPM) was observed with NAC and AST (Fig. 10C, F). The similar pattern observed with NAC and AST suggests that the shared antioxidant properties of the two treatments prevented the effects of prenatal stress on the embryonic/neonatal brain of both sexes. Both antioxidant treatments are known to restore antioxidant enzymes, such as glutathione peroxidase (Mukherjee et al., 2007; Leite et al., 2010). Male and female embryos were found to have similar activity levels of glutathione peroxidase (GPx1) in the embryonic forebrain, so samples were pooled and sex-balanced and reported as a single group. The results from Aim 1.3 indicate that prenatal stress trend decreases the activity level of GPx1 and NAC significantly increased the activity by 196.5% (Fig. 6D). Further illumination of this fetal programming mechanism might show that GPx1 activity plays a role in the effects of prenatal stress on anxiety-like behaviors. Further exploration of these mechanisms might involve administering both treatments simultaneously (a cocktail of antioxidants to the mother) to examine the effects a double-hit of antioxidants has on antioxidant enzyme activity in the embryonic brain.

On the other hand, prenatally-stressed male and female offspring exhibited impaired motor learning on the rotarod task and neither NAC nor AST prevented the impairments (Fig. 15). However, unlike anxiety-like behavior, the neural correlates and neural circuitry of motor learning is not associated with the medial front cortex (mFC) and the hippocampus, but with the cerebellum and basal ganglia. Those two regions of the brain are not populated with medial ganglionic eminence-derived GABAergic interneurons. Therefore, differences observed in the
motor learning between prenatally-stressed and non-stressed mice might not be due to redox dysregulation, but other cellular mechanisms. These mechanisms should be explored in future experiments.

The purpose of Aim 2 was to investigate the effects of prenatal stress on behavior and postnatal GABAergic neuron populations in adult offspring brain. We found evidence that redox dysregulation mediates the effect of prenatal stress on anxiety-like behavior in male and female offspring mice and sensorimotor gating in male adult offspring. That is to say that maternal antioxidant treatment prevented the deficits observed in the prenatally-stressed control group (PBS), and, in the case of female mice, NAC and AST alone improved sensorimotor gating and NAC alone improved habitual learning and cognitive flexibility. It is important to note here that there are divergent results between the two treatments in a couple of behavioral assay for the female offspring. For example, only NAC improved the habitual learning and cognitive flexibility in female mice on the water T-maze task (WTM). It could be that our antioxidant treatments are affecting different developmental pathways and that only NAC influenced pathways that would develop into behavioral outcomes that affected habitual learning and cognitive perseverance on the WTM. For example, NAC is commonly used in clinical studies as a glutamatergic modulator to treat obsessive-compulsive disorders (Chakrabarty et al., 2005). NAC is converted to cysteine, a substrate for the glutamate-cysteine antiporter, and this antiporter works to reverse transport glutamate into the extracellular matrix, thereby reducing synaptic release of glutamate (Grant et al., 2009). It could be that NAC, which is commonly used for treating obsessive-compulsive disorders (OCD), prevented cognitive perseverance in our female mice—something that goes hand-in-hand with OCD.
While we found evidence of NAC and AST preventing some prenatal stressed-induced behavioral outcomes, we did not find evidence that redox dysregulation was involved in the prenatal stress-induced impairments in sociability in female offspring mice, cognitive inflexibility of male offspring mice, and deficits in motor learning in male and female offspring mice. Namely, maternal antioxidant treatment did not prevent impairments that characterized the prenatally-stressed group. We also found evidence that prenatal stress did not affect social novelty and activity levels in male or female offspring mice, sensorimotor gating and habitual learning in female mice, and habitual learning in male offspring mice. This study also highlights another complicating factor in our interpretation of the results. We did not find any sex differences in the assays performed on our embryonic brains, but we found several instances of sex differences in the behavioral assays and the ratio of PV+/GAD67+ cells in the mFC. Because we did not observe sex differences in GABAergic progenitor cell migration, redox-related gene expression, and antioxidant enzyme activity (all of Aim 1), we can assume that the sexually dimorphic effects on cortical interneuron populations must occur after our experimental window of observation (E14).

One inevitable problem with studying redox dysregulation is the issue of an imbalance in the pro-oxidant-antioxidant homeostasis, which suggests that problems could arise from perturbation on either side of the equilibrium. A delicate balance between the reductive and oxidative states is crucial for normal cellular metabolism. Consequently, there is a biological goldilocks zone of relative levels of ROS wherein excess levels of ROS are equally as detrimental as insufficient levels of ROS at critical periods of development (Castagné et al., 1999). An additional problem is attempting to study redox dysregulation during embryonic development since ROS are vital to normal embryonic development through cellular signaling.
and regulation of cellular fate (for an extensive review on the subject, please see Dennery (2007)). Therefore, the antioxidant rescue from Aim 1.4 should be interpreted with this caveat in mind. This study recapitulated the findings of Stevens et al. (2013) and Gumusoglu et al. (2017b) that demonstrated that prenatal stress delayed inhibitory progenitor cells into the neocortex of E13 embryos. We found that when maternal antioxidant treatment was given right before prenatal stress, prenatally-stressed and non-stressed embryonic brains showed a “rescue” of this delay (Fig. 8A-G). However, the rescue of this delay phenotype was overcorrected, evidenced by the GAD67GFP+ cells migrating farther along the migration stream than the non-stressed control embryos (Fig. 8G). In other words, we may have overshot the target with our NAC and AST dosage. Because both too much and too little cellular ROS can have damaging effects (Le Belle et al., 2011) in the embryonic brain, this study underscores a significant problem with developing effective therapeutic interventions with the purpose of restoring a normal redox balance in the developing brain.

The results of this dissertation thesis are noteworthy because they elucidate one of the mechanisms by which prenatal stress affects the development of inhibitory systems in the embryonic brain. Several studies have shown the prenatal stress has a causal role in the neural circuitry in the mature brain and adult psychopathology (Huot et al., 2004; King and Laplante, 2005; Weinstock, 2008; Bale, 2009; Harris and Seckl, 2011; Weinstock, 2017). Numerous studies have also shown that redox dysregulation contributes to neurological disorders (Peuchant et al., 2004; Cambonie et al., 2007a; Derks et al., 2010b; Ziech et al., 2011). These sets of experiments are the first to investigate whether perturbations to the redox state act as an intermediary between the effects of prenatal stress and altered GABAergic development in the embryonic and fetal brain. It is my hope that these results and conclusions will address these
questions and fill the gap in our knowledge regarding the cellular and molecular mechanisms that are modified by prenatal stress. The ultimate goal is to advance our knowledge of the risk factors for mental illness, including genetic, biological, and environmental causes and, equipped with this knowledge, develop better treatments and interventions for those at risk for psychiatric disorders.
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