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Interactions Between Chemical Stressors and Social Factors that Impact Children's Health and Development

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Assessing Environmental Health Disparities in Vulnerable Groups: Interactions Between Chemical Stressors and Social Factors That Impact Children's Health and Development

by

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Executive Summary

This report summarizes research conducted to investigate how non-chemical stressors may modify responses to chemical exposures leading to untoward changes in susceptible and vulnerable subpopulations, with a focus on children. All studies and the findings mentioned in this report have been previously published in highly regarded toxicological peer-reviewed journals. The objectives of the research were to provide information responsive to the needs of public health officials in optimizing their community environments and developing sound strategies to improve children's health and mitigate health disparities. Two approaches were undertaken to meet these objectives: (1) laboratory research with rodent models, and (2) literature reviews and analyses.

Laboratory Studies: Two experimental models using rats were employed to evaluate interactions between chemical and non-chemical stressors: (a) effects of maternal obesity induced by a high-fat diet during pregnancy on responses to the air pollutant ozone (O_3) in offspring; and (b) effects of maternal psychosocial stress on manganese (Mn)-induced alterations of neurobehavioral development in offspring.

Results from our studies indicated an age-related pattern in pulmonary effects of O_3 exposures, with adolescent and young adult animals being more susceptible to altered respiratory function, and induction of lung cell inflammation and injury. Dams fed a high-fat diet one month before mating and throughout gestation and lactation had increased body weight and body fat during pregnancy. Similarly, body weight and body fat of the offspring were elevated and persisted postnatally despite cessation of dietary exposure after weaning. At 90 days of age, O_3 exposure caused exaggerated alterations in pulmonary function and lung injury, as well as gut microbiome and metabolic function in the obese offspring compared to non-obese controls. Our findings suggest that maternal high-fat diet and obesity enhance the susceptibility of offspring to environmental stressors.

To examine the interactions between maternal psychosocial stress and exposure to Mn in drinking water, our objective was to produce *chronic low-level stress* in the rat to simulate those experienced in people living under socioeconomic and environmental disadvantages. Using animal models to evaluate responses to chronic low-level stress poses technical challenges because rodents are adept at habituating to recurring stressors. Accordingly, a model of multiple non-invasive stressors was designed in our laboratory to elicit and maintain a mild stress response in rats during pregnancy. Maternal exposure to Mn in drinking water led to a dose-dependent increase of this metal in the brain of rat offspring, which was unaltered by maternal stress. However, Mn altered several measures of neurobehavioral development. Motor output and affective states determined by locomotor activity and thigmotaxis (the tendency to move toward physical contact) were altered only by Mn in the offspring; these effects were not seen with maternal stress alone or in combination with chemical exposure. In contrast, cognitive functions including short-term memory, learning, attention, impulsivity, and reaction time were altered by Mn or maternal stress alone, but the combination of these two factors exerted a different response profile. Rather than an additive response, an attenuation or reversal of effects was seen, raising the possibility that maternal stress may mask the insults of some developmental neurotoxicants.

Literature review:

Three topical literature reviews were conducted to support the tenets of this research: (a) epigenetics and the developmental origins of health and disease; (b) epigenetic consequences of maternal smoking during pregnancy, and latent health effects in offspring; and (c) prenatal chemical exposure and the risk of childhood cancer.

There is now compelling epidemiological and laboratory experimental evidence that the in utero and early postnatal environments affect lifelong health and disease susceptibility. Evidence supporting influences of maternal diet on the epigenome of her offspring is strong, and exposure to chemical and nonchemical stressors (e.g., psychosocial stress) during pregnancy can also have similar long-term effects on offspring. Findings that support epigenetic programming by chemical and nonchemical stressors, including vinclozolin, bisphenol A, metals, therapeutic drugs, maternal behaviors, and assisted reproduction technologies were summarized in the first review.

The best example of an exposure during pregnancy in humans resulting in epigenetic changes and elevated risk of metabolic disease in offspring is maternal smoking. Maternal smoking causes lower birth weight, birth defects, and other adverse pregnancy outcomes. The latent and persistent metabolic effects in offspring of smoking mothers resemble those observed in studies of maternal undernutrition during pregnancy. Indeed, persistent altered patterns of DNA methylation have been documented in smoking mothers' offspring and are related to adverse health outcomes including low birth weight.

Lastly, the issue of whether children are more vulnerable than adults to carcinogens, including in utero exposure, was examined. Potential mechanisms of prenatal cancer induction, including the emerging concept of epigenetic programming during early life was discussed. Several case studies highlighting diverse prenatal exposures that increase cancer risk later in life, including radiation, diethylstilbestrol, tobacco smoke, pesticides, and arsenic, and incidence of specific cancer types, such as breast cancer and leukemia, were described. There is ample evidence from both human and experimental animal studies to support the idea that prenatal exposure to carcinogens is sufficient to induce cancer later in life in offspring. However, the prenatal period may be more, similarly, or less sensitive to the induction of cancer from chemical exposures than the adult, depending on the nature of the carcinogen.

In summary, findings from this research provide information to help public health officials and community leaders optimize environments and develop sound strategies for children's healthy development and mitigate health disparities derived from exposures to environmental pollution and modified by non-chemical factors. Although epigenetic changes were not determined in the laboratory studies, it is clear from the literature reviews that this is a plausible and potentially powerful mechanism underlying adverse effects from exposures to chemical and nonchemical stressors during development. Accordingly, evaluation of these changes should be considered as key events in adverse outcome pathways for chemical and non-chemical effects on growth, physiology, and metabolism of offspring in future investigation.

Section 1. Introduction

1.1 Background.

Human health and well-being are inextricably linked to the environment and can be impacted by exposure to environmental pollutants and non-chemical socioeconomic factors (e.g., dietary imbalance, psychosocial challenges) at various life stages. At EPA, research was conducted to focus on how non-chemical stressors may modify responses to chemical exposure, leading to untoward changes in health conditions for susceptible and vulnerable groups including infants and children. The objectives of the research were to provide information to aid public health officials in optimizing their community environments and developing sound strategies to improve children's health and mitigate health disparities. Two approaches were undertaken to meet these objectives: (1) laboratory research with rodent models, and (2) narrative and literature reviews. This report is a synopsis of our experimental approaches and results from these studies. Details of specific findings can be gleaned from the individual published papers listed in Appendix A.

Children are a vulnerable group based on life stage; they have different activities, exposure scenarios and diets, as well as developmental and physiological differences compared to adults. To improve children's health in communities, considerations must be given to the total environment: where they live, what they eat, and their types and levels of activity, as well as their total exposome, including lifetime exposure history. A child's environment starts in utero, so similar considerations of the mother's health and total environment are also important. The susceptibility of children's exposure to maternal stressors during development or early childhood often is expressed (or even amplified) when they are challenged with further exposure to the same or new environmental stressors during childhood or later life.

Differences in environmental quality between wealthy and economically challenged communities can affect the health and well-being of children living in those locales. Such so-called "health disparities" can begin in utero, at the earliest stages of development, and extend or possibly worsen during childhood and adolescence. Stressors in poor quality environments may include chemical (environmental contaminants) as well as non-chemical factors such as lack of access to natural enrichments (e.g., urban greenways and recreational areas), deteriorating elements of the built environment, social factors (e.g., poverty and crime), and health factors (e.g., maternal stress and poor maternal or child nutrition). These non-chemical factors can contribute to the manifestation of health disparities in children. Importantly, most, if not all, of these factors, are modifiable by improving the environment. This research entails the use of laboratory animal models comprising several maternal conditions that are common among disadvantaged groups of humans, and which may have deleterious effects on children's development and exacerbate the adverse effects of exposure to environmental pollutants.

The underlying tenet of this research is the concept of "Developmental Origins of Health and Disease" (DOHaD), which evolved from the "Barker Hypothesis" first proposed in the 1990s (review: Barker, 2007). The hypothesis states that the structure and function of bodily organs, endocrine controls of physiology, homeostasis, and energy balance undergo "programming" in response to the intrauterine and early life environment. This programming determines, in part, the set-points of physiological and metabolic responses that persist throughout life. For instance, alterations in embryonic and fetal

nutrition, as well as endocrine status during gestation, have been shown to result in developmental adaptations that produce permanent structural, physiological, and metabolic changes. These changes can predispose an individual to cardiovascular, metabolic, and endocrine diseases later in life, including type 2 diabetes and obesity, conditions that are rapidly rising among children and adults in the United States and around the globe.

The seminal findings by Barker and colleagues originated from epidemiological surveys demonstrating that the incidence of coronary heart disease in later life was correlated inversely to birth weight, even within the normal range. This correlation was independent of age, sex, ethnicity, socioeconomic status, and marital status. Subsequently, an inverse relationship between birth weight and incidence of impaired glucose tolerance or diabetes was observed, leading to the "Thrifty Phenotype" hypothesis, which states that a malnourished fetus makes adaptive changes in energy metabolism, including reduced insulin secretion and insulin resistance, to improve survival under conditions of nutritional deprivation (Hales and Barker, 2001). Gluckman and Hanson (2006) further refined this concept, calling it a "predictive-adaptive" response by the fetus to low nutrient availability by conserving energy, i.e., storing fat. Adaptations to low nutrient availability become maladaptive if the postnatal environment is one of abundant nutrition, such that the risk of obesity and related disease risk are elevated.

Although the initial focus was cardiovascular and metabolic diseases, the Barker Hypothesis has been extended to other adult disease risks, including effects on kidney size and function, lung function, immune function, learning ability, mental health, aging, and cancer, although associations tend to be less consistent than those for coronary heart disease and diabetes.

Mechanistic investigation of developmental programming is still in its infancy, although several key players have been proposed. These include hormones that regulate fetal growth and development, such as insulin, insulin-like growth factors (IGF-I and IGF-II), prolactin, thyroid hormones, and glucocorticoids. These hormones may act as nutritional and maturational cues that adapt fetal development to the prevailing intrauterine conditions, thereby maximizing the chances of survival in utero and after birth if those conditions persist.

The emergence of epigenetic research in the past few decades has provided crucial insights into the regulation of growth and development and biological mechanisms underlying DOHaD processes. Epigenetic control of gene expression involves modification of the genome without altering the DNA sequence and is typically mediated by changing the DNA methylation pattern and/or modifications of chromatin packaging via acetylation, methylation, or phosphorylation of histone proteins.

The epigenome is established at specific stages of gametogenesis and embryo/fetal development, after which it is largely maintained throughout life. Thus, the embryo and fetus are uniquely vulnerable to reprogramming based on maternal nutrition, physiological conditions, and exposure to xenobiotics (drugs and environmental pollutants). For example, the pioneering work of Waterland and Jirtle (2003) demonstrated that dietary supplementation can alter dramatically a heritable phenotype in mice based on epigenetic changes in DNA methylation. Many genes potentially involved in fetal programming have been determined to be under epigenetic regulation, including the glucocorticoid receptor, proopiomelanocortin, 11β-hydroxysteroid dehydrogenases, corticotrophin-releasing factor, leptin, glucose transporter, and peroxisome proliferator-activated receptors. Hence, it is reasonable to hypothesize that epigenetic alterations of gene expression in response to the developmental milieu may influence the developmental programming of metabolism. Indeed, a review of the recent

advances of epigenetics and their application to the understanding of developmental origins of health and disease has been published as part of our research efforts (Rogers et al., 2018).

Although nutritional aspects dominated early DOHaD research and continue to remain a primary focus in the field, the concept has captured the attention of developmental toxicologists. A review of the environmental epidemiological literature by Heindel et al. (2017) noted recent publications, covering over 60 different chemicals, and focusing on neurological, cancer, and respiratory outcomes in children and young adults after early life exposures. Similarly, toxicological research with animal models has examined adverse effects of specific chemical exposures during pregnancy on adult health (e.g., Grun and Blumberg, 2007; Dolinoy et al., 2006, 2007; Rogers et al., 2014; Howard, 2018; Young and Cai, 2020). More recently, Rumrich et al. (2020) proposed that DOHaD considerations should be broadly incorporated in health risk assessment by regulatory agencies.

In this report, we summarize how both laboratory and epidemiological research can be used to expand our understanding of effects of chemical and/or non-chemical stressors in the environment on fetal development and latent disease risks. Ultimately, we hope that the application of this research will be instrumental in improving human health risk assessment of chemicals in the context of the total environment (physical and social), and thereby aid in developing policies to improve community health and promote environmental justice.

1.2 Laboratory research

Two studies were conducted to examine how nonchemical stressors modify adverse outcomes from exposures to environmental pollutants, such as those experienced by residents in at-risk communities (e.g., those in economically disadvantaged neighborhoods or near contaminated sites). Obesity is a condition that has reached epidemic levels in our nation, primarily as a result of high-calorie, high-fat diets and sedentary lifestyle. One study evaluated interactions between hypercaloric-diet-induced maternal obesity and postnatal exposure to ozone in rodent offspring. Chronic low-level stress associated with socioeconomic and environmental disparities have been linked to detrimental mental health outcomes. The second study examined interactions between mild behavioral stressors (to mimic psychosocial stress) and perinatal exposure to manganese in drinking water on neurobehavioral development of rodent offspring.

1.3 Review of the literature

Three literature reviews focusing on the developmental aspects of diseases associated with chemical exposure at early life stages were conducted to support this research: (1) epigenetics and the developmental origins of health and disease; (2) smoking and pregnancy: epigenetics and the developmental origins of metabolic syndrome; and (3) developmental origins of cancer.

1.4 Outline of report

This report is comprised of five sections. In addition to this section, Section 2 describes the interactions between maternal obesity and ozone exposure in rat offspring at different life stages and strategies to mitigate the adverse health outcomes. Section 3 describes interactions between maternal psychosocial stress and prenatal exposure to manganese on neurological development of rat offspring. Section 4 highlights the salient findings from three literature reviews on epigenetics as a plausible mechanism underlying adverse developmental health outcomes derived from interactions between chemical and

nonchemical stressors, adverse developmental outcomes from maternal cigarette smoking, and origins of childhood cancer. Lastly, Section 5 provides a summary and conclusions of findings described in this report.

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Section 2: Social and personal vulnerabilities modify the health impact of environmental exposures

2.1. Introduction

The incidence of metabolic disorders has increased worldwide. The U.S. Centers for Disease Control and Prevention's 2015-2016 report indicates that 38.9% of U.S. adults and youth are obese. In 2015, an estimated 30.3 million people of all ages in the U.S. population (9.4%) had diabetes; prevalence was higher in American Indians/Alaska Natives, non-Hispanic Blacks, and people of Hispanic ethnicity than among non-Hispanic whites (CDC, 2020). Several lifestyle risk factors, such as calorie-rich diets, large portion sizes, low activity levels, and increased psychosocial stress, have been implicated in the increasing prevalence of metabolic and other chronic diseases. The epidemic growth in the incidence of obesity, type 2 diabetes, and related metabolic diseases in the United States and other countries is thought to be primarily due to genetics combined with increased consumption of diets containing high quantities of fat and sugars (Stott and Marino, 2020; Kadayifci et al., 2019; Lichtveld et al., 2018). Consumption of Western type diets with high fructose and fats with low essential nutrients has been associated with metabolic syndrome (Bray et al., 2004; García-García et al., 2020; Nagao et al., 2015).

Unhealthy diets are detrimental to health for all life stages, including during pregnancy and development. Maternal high-fat diet has been associated with greater incidence of obesity, diabetes, and other inflammatory diseases in children (Shankar et al., 2017). Sex-dependent effects of maternal obesity are linked to impairment of insulin, glucose, and lipid metabolism in multiple tissues of rat offspring (Lomas-Soria et al., 2018). It is postulated that circulating lipid metabolites from obese mothers likely influence the fetal metabolic phenotype during development (Lewis and Desoye, 2017). Transcriptomic assessment of baboon fetal livers born to mothers fed a high-fat/high-fructose diet indicated dysregulation of the tricarboxylic acid cycle and glycolysis, changes in Wnt/β-catenin signaling, and marked lipid accumulation (Puppala et al., 2018). Epigenome-wide methylation changes (Hjort et al., 2018) and hypothalamic leptin and insulin resistance are postulated to be contributing factors (Gomes et al., 2018). These studies provide evidence that maternal diet and obesity are risk factors for offspring metabolic programming.

Dietary supplements high in omega-3 and omega-6 unsaturated fats, and several other specialty foods are used widely for improving health. The beneficial effects of dietary polyunsaturated fatty acids have been noted in diabetes (Brown et al., 2019), cardiovascular disease (Abdelhamid et al., 2018), kidney diseases (Syren et al., 2018), major depressive disorders (Husted and Bouzinova, 2016), and other inflammatory conditions (Calder, 2017). Several studies have examined health benefits of these dietary supplements in ameliorating the effects of air pollution (Lin et al., 2019; Tashakkor et al., 2011). However, as with any other diet, the quantity being consumed can be a critical determinant of health benefits with these dietary supplements (Desnoyers et al., 2018).

Because an organism's growth and physiological functions require regular intake of food, it is likely that human health and the responses produced by environmental stressors can be impacted by diet at multiple levels, including the gut-brain axis, neural regulation, metabolism, and immune response. Diet-induced health conditions may create unique susceptibility in individuals when environmental stressors are encountered. For example, diabetes, obesity, and hypertension may exacerbate inflammation resulting from exposure to air pollution (Dubowsky et al., 2006). Thus, these

susceptibility factors need to be considered when evaluating factors impacting health during exposure to environmental stressors. Despite its prevalent consumption, there are relatively few studies demonstrating a link between consumption of an unhealthy diet high in carbohydrates and fat and susceptibility to environmental stressors. Wagner et al. (2014) recently demonstrated that feeding rats a high-fructose diet to induce a metabolic syndrome led to an exacerbation of bradycardia induced by exposure to particulate matter and ozone. There is evidence of altered susceptibility to air pollutants, such as ozone in genetic animal models of obesity (Shore et al., 2009; Dye et al., 2015). Diet-induced models of obesity also have suggested the same link, but there is little information on the role of gender on the response to ozone or other air pollutants.

Researchers at EPA conducted experiments to examine how carbohydrate- or lipid-rich diets and popular dietary supplements may alter responsiveness to environmental stressors, such as air pollutants (other studies reviewed in Whyand et al., 2018) in collaboration with the Air and Energy research program. These studies addressed how the combination of lifestyle factors, including level of activity, unhealthy and healthy diets, age (including prenatal and postnatal periods), and underlying metabolic disease, interacts with an environmental stressor (ozone) to affect health in laboratory animals. Ozone as a prototypic environmental stressor was selected because it has been shown to induce pulmonary and systemic effects through a classical neuroendocrine stress pathway that also is linked to neuropsychiatric and peripheral chronic diseases, including metabolic derangements induced by nonchemical stressors. Given looming mental health crises, increased social vulnerabilities due to the pandemic, and climate-related increases in ambient temperatures, the understanding of cumulative health burden resulting from environmental and non-chemical stressors becomes critical, especially for communities with inequalities (Environmental Justice). In order to begin understanding which underlying conditions are most important, and how they may modify the responses to environmental exposures, experimental studies were conducted specifically considering ozone as a prototypic air pollutant, are depicted below. The choice of ozone is based upon the evidence from EPA that ozone's effects are mediated through neuroendocrine system, the very system that is involved in mediating health effects for many "non-chemical" physiological and psychosocial stressors.

2.2 Life stage and susceptibility to ozone-induced pulmonary health effects

One of the goals of the Sustainable and Healthy Communities program has been to assess the susceptibility of children and the elderly to environmental insults. Studies have been conducted in animal models to determine how biological responses to environmental stressors differ at different life stages. Neuroendocrine and metabolic effects of acute and subchronic ozone exposure are more prominent in young animals when compared with the old (Bass et al., 2013), whereas recovery from ozone effects generally is delayed in older animals, suggesting a compromised coping response. Particularly for respiratory effects of ozone, old animals had more persistent effects (Gordon et al., 2013, 2014). Snow et al. (2016) described the age-related differences in pulmonary effects of acute and subchronic ozone exposures in rats. For example, age-related differences in breathing frequency indicated that young animals breathe much faster than do older animals, resulting in different patterns of deposition in the lung after an exposure to air pollution, which may result in higher inhaled ozone dose in young animals and more severe lung inflammation, as we observed (Figure 2.1; Figure 2.2).

Figure 2.1. Whole-body plethysmography performed on rats exposed to filtered air or Ozone for 6h/day for 2 days (acute) or 2 days/week for 13 weeks (subchronic).

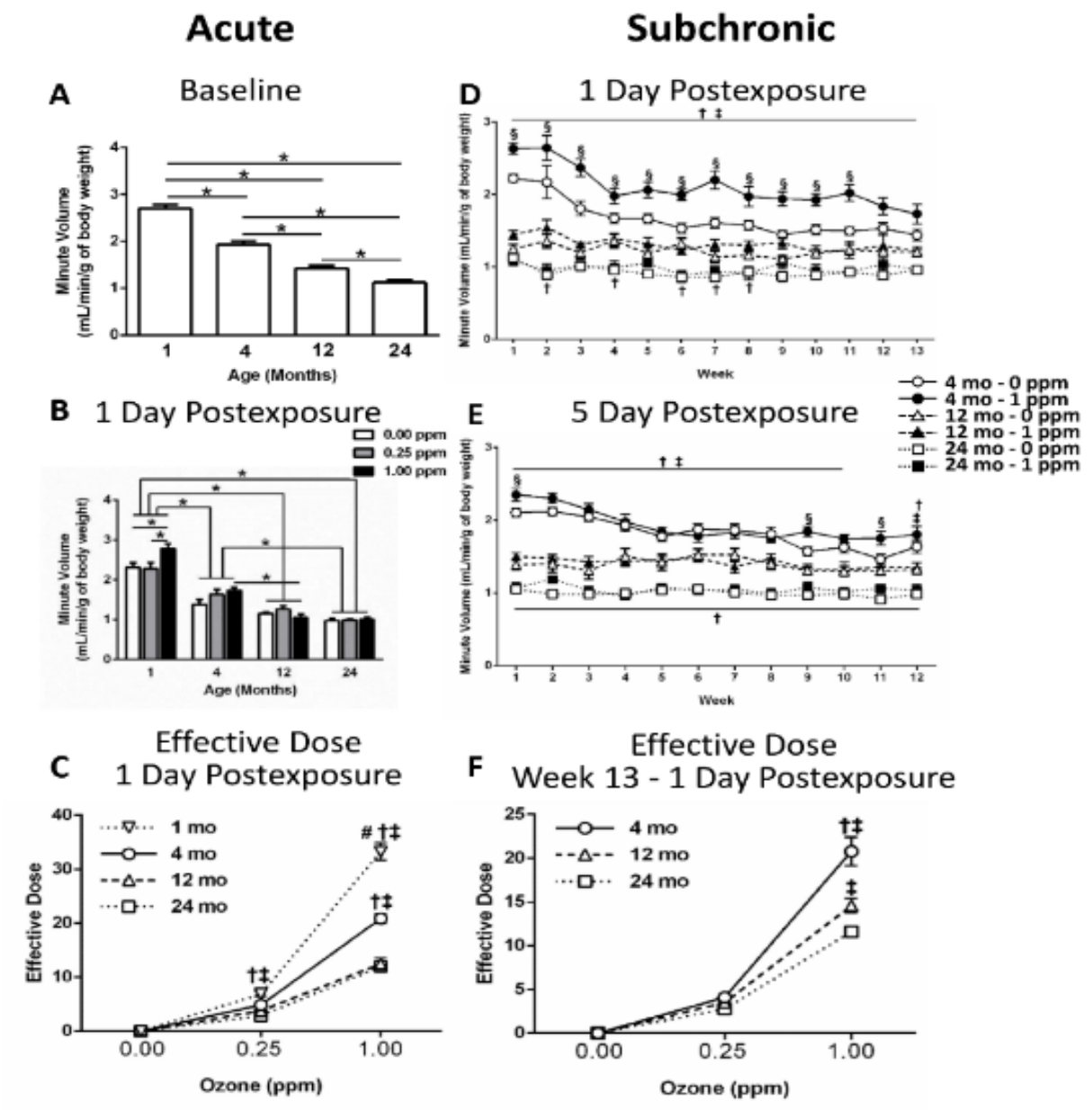


Figure 2.1. Whole-body plethysmography was performed on rats that were exposed to filtered air or O_3 for 6h/day for 2 days (acute) or 2 days/week for 13 weeks (subchronic). Measurements for minute volume were taken (A) at baseline prior to the acute exposure (n=18-24), (B) 1 day postexposure in the acute group (n=6-8), (D) weekly at 1 day postexposure in the subchronic group (n=8-10), and (E) weekly at 5 days postexposure in the subchronic group (n=8-10). Effective dose (O_3 ppm x h x minute volume) was calculated for (C) the acute group (n=6-8) at 1-day postexposure and (F) the subchronic group (n=8-10) for week 13 at 1 day postexposure. Data show mean ± SE. * = $p<0.05$ significantly different between groups. § = $p<0.05$ significantly different between 0- and 1- ppm groups of the same age. # = $p<0.05$ significantly different than the 4-mo animals. † = $p<0.05$ significantly different than the 12-mo animals. ‡ = $p<0.05$ significantly different than the 24-mo animals. (Reprinted from Snow et al., Inhal Toxicol. 2016;28(7):313-323).

After weekly exposure of young and senescent rats to ozone for 15 consecutive weeks, it was apparent that ozone effects on minute volume was maximum for 1-mo old animals. The increased minute volume can result in higher retained dose of ozone in the lung. Greater sensitivity of young animals to acute ozone exposure also is reflected in the inflammatory response induced by ozone in young versus old rats, as evidenced by increased presence of neutrophils in bronchoalveolar lavage fluid (Figure 2.2).

Figure 2.2. Lung neutrophilic inflammation induced by exposure to Ozone 6h/day for 2 days (acute) or 2 days/week for 13 weeks (subchronic).

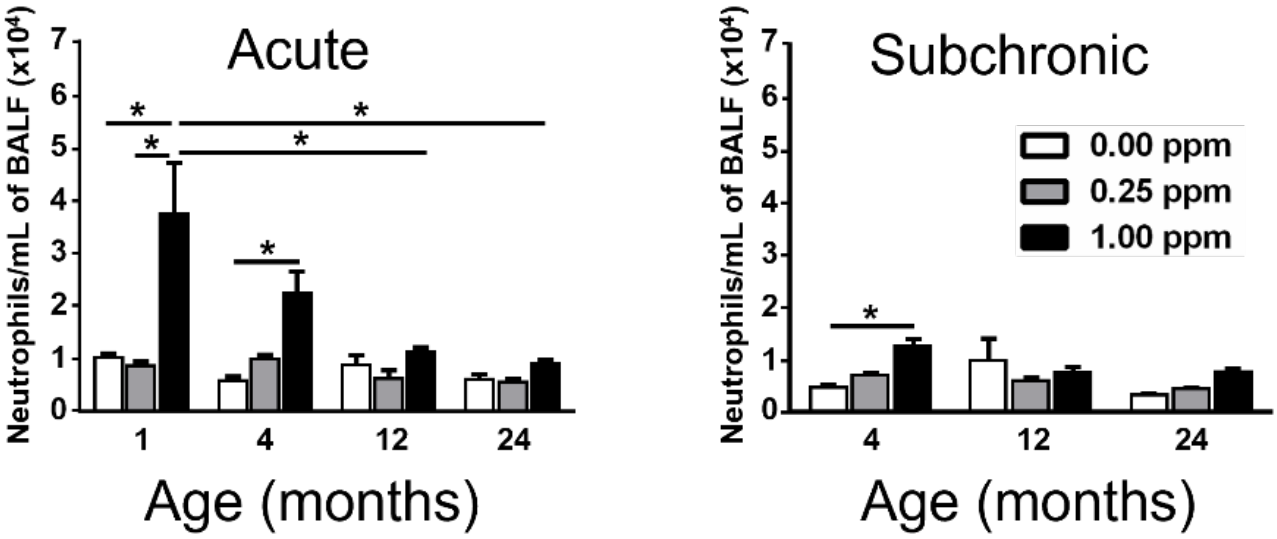


Figure 2.2. Lung neutrophilic inflammation induced by exposure to ozone 6h/day for 2 days (acute) or 2 days/week for 13 weeks (subchronic). Asterisks denote $p < 0.05$ significantly different between groups. (Reprinted from Snow et al., Inhal Toxicol. 2016;28(7):313-323).

Assessing breathing parameters enabled us to determine the effective ozone dose to the lung, which showed that, at all concentrations of ozone, the effective lung doses were highest for 1-mo old animals when compared with old animals, suggesting that children may encounter greater ozone doses to the lungs and may experience larger health burdens from ozone exposure (Figure 2.2). Age of 1-mo in rats mimics age of a toddler nearing 3 years of age in humans (Sengupta, 2013). In conclusion, adolescent and young adult rats were found to be particularly susceptible to acute ozone-induced changes in respiratory function, induction of lung cell injury, and neutrophilic inflammation.

Age-related susceptibility and increased sensitivity of young animals to acute ozone exposure is not restricted to lung tissue, as other organs also are affected (Bass et al., 2013; Gordon et al., 2013). We examined age-related and subchronic ozone-induced changes in markers of oxidative stress in brain regions of rats. Air-exposed aged rats had increased oxidative stress in the frontal cortex, striatum cerebellum, and hippocampus compared with young rats, consistent with human data showing age-related increased oxidative stress associated with decrements in antioxidant homeostatic mechanisms (Gámez-Valero et al., 2020). However, our study showed that when young and aged rats were exposed to ozone, the young rats experienced greater increases in oxidative stress in selected brain regions than did aged rats (Kodavanti et al., 2021). Taken together, these results indicate that oxidative stress may be increased in brain regions after subchronic ozone exposure, but the complex interactions

among age, exposure, and brain region may be dependent on the existing antioxidant reserve. Thus, children exposed to higher levels of ozone at younger age might also be more susceptible to chronic neural diseases such as Alzheimer's and/or Parkinson's at later age. Indeed, long-term ozone exposure has been shown to dysregulate the amyloid plaque microenvironment in a mouse model susceptible to Alzheimer Disease-associated pathology (Greve et al., 2022). Longitudinal epidemiological studies will need to factor in the childhood-related environmental exposure to determine potential linkages to brain disorders.

2.3 Active lifestyle and reduction of ozone-induced pulmonary and systemic effects

A sedentary lifestyle may make humans more susceptible to environmental insults. Given the pandemic-related shift in increased teleworking, the risk factors related to sedentary lifestyles are likely to be worsening for coming years. EPA researchers examined whether exercise during childhood offers protection against ozone-induced pulmonary and metabolic effects in young adults. Likewise, it was presumed that young animals allowed to run on training wheels relative to those laboratory-housed animals without the training wheel may represent active versus sedentary lifestyle in humans, and that the sedentary animals might experience more detrimental effects of ozone exposure when compared with active animals. Gordon and collaborators (2017a) conducted an experiment in which young female Long-Evans rats were housed individually in rodent cages with or without a running wheel while they grew up (Figure 2.3).

Figure 2.3. Training/exercise in rats is associated with loss of body fat.

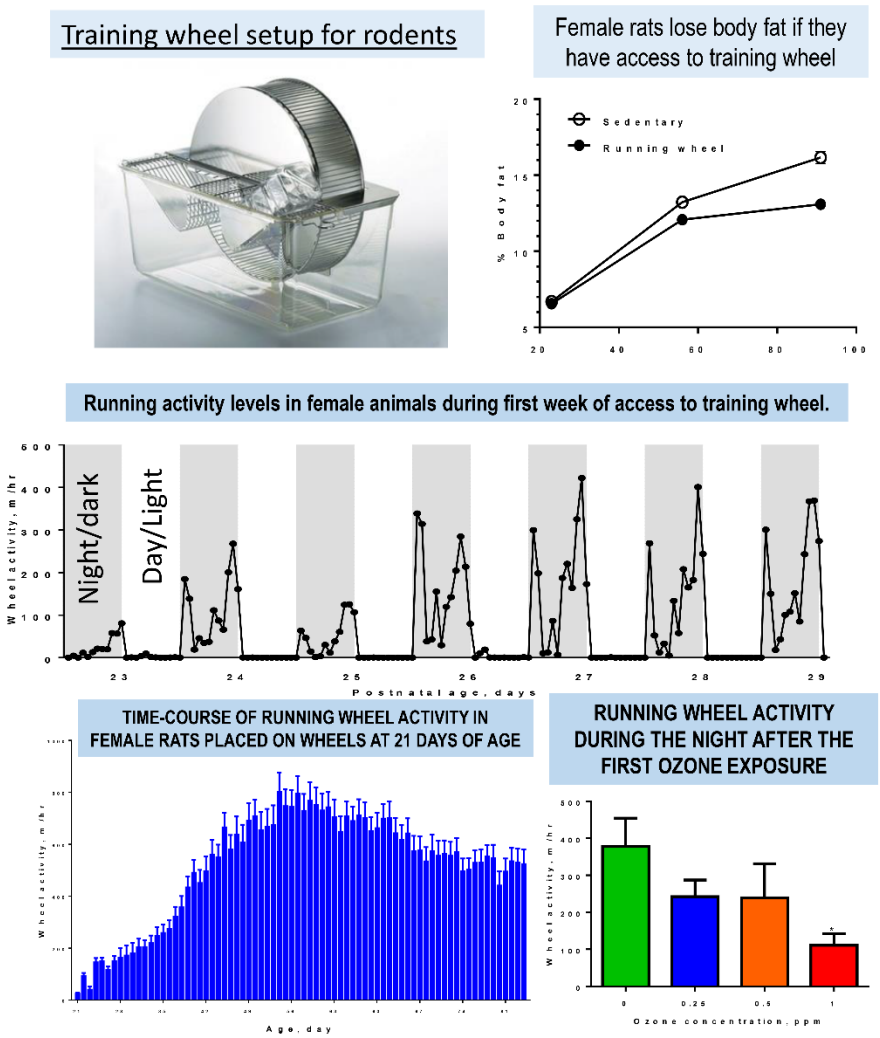


Figure 2.3. As in humans, training/exercise in rats is associated with loss of body fat. Rats are active during nighttime when they run on wheels. Data obtained from Gordon et al. (Am J Physiol Lung Cell Mol Physiol. 2017;312(1):L100-L109 and Inhal Toxicol. 2016;28(7):293-302).

It was noted that active rats maintained a more favorable body fat percentage when compared with sedentary rats. Access to a running wheel improved glucose sensitivity at baseline, demonstrating positive metabolic effects of increased physical activity in rats.

Furthermore, after 12 weeks of training, rats were exposed to air or one of three concentrations of ozone (0.25, 0.5, or 1 ppm) for 5 h/day for 2 consecutive days to determine if an active lifestyle reduced lung injury and systemic metabolic effects of ozone. Our prior studies have shown that acute ozone exposure induces glucose intolerance through the activation of neuroendocrine-mediated release of adrenal-derived stress hormones (Bass et al., 2013; Miller et al., 2015). Ozone-induced glucose intolerance was lower in animals with access to a running wheel relative to those without one. The animals that ran had attenuated ozone-induced increases in BAL eosinophils following exposure to 1 ppm and 0.5 ppm ozone, suggesting increased allergic response. However, other adverse effects of

ozone, such as protein leakage and neutrophilic inflammation, were not affected by exercise, suggesting that further research is needed to understand the contributing factors.

Physical activity also appeared to protect brain tissues from ozone toxicity in animals. Although more reactive microglia were found within the hippocampus of animals exposed to ozone in both sedentary and active rats, mitochondrial bioenergetic parameters in the hypothalamus were affected significantly by exercise, suggestive of increased energy production. Mitochondrial complex II activity in the hippocampus was affected significantly by both exercise and ozone exposure. It was demonstrated that ozone exposure induced microglia reactivity within stress centers of the brain and altered mitochondrial bioenergetics. Some of these effects were reduced by exercise, suggesting a role for lifestyle in mitigating ozone effects on brain mitochondrial parameters, in agreement with the authors' previous reports on other endpoints (Valdez et al., 2020). Collectively, these data provide experimental evidence of lifestyle factors modifying ozone effects. However, it is not clear if this was true for all biological responses related to metabolic or immune processes. Other environmental stressors, besides air pollutants, might elicit unique susceptibility in sedentary individuals; however, such experimental evidence is currently limited. Sedentary lifestyle is an important component of social vulnerability and community level assessment could help determine coherency between experimental data and human observational studies.

2.4 The influence of high fructose, high-fat diet on ozone pulmonary and systemic effects

Communities that consume unhealthy diets rich in sugars, starch and lipids are more vulnerable to environmentally induced diseases. High fructose corn syrup and saturated fats make up a large portion of calories in these unhealthy diets, contributing to metabolic diseases such as obesity and diabetes. It is well established that economically disadvantaged individuals are more likely to consume unhealthy diets and are also likely to have greater exposure to environmental pollutants because of their occupation and/or residence being in more congested areas or near major industrial sites (WHO, 2004). These scenarios were modeled in several animal studies that examined the influence of dietary factors on the pulmonary and systemic toxicity of ozone.

The influence of high-fat or high-fructose diets on ozone-induced lung injury and inflammation was examined (Gordon et al., 2016a). We hypothesized that high-fructose and high-fat diets would exacerbate the toxic effects of ozone, especially effects on metabolic endpoints. Male and female Brown Norway rats received control, high-fat, or high-fructose diets starting at 4 weeks of age. At 12 weeks of age, rats were exposed to air or 0.25, 0.5 or 1.0 ppm ozone for 1 day (5 h, acute) or for 1 day/week for 4 consecutive weeks (subchronic). Acute and subchronic ozone effects in the animals fed the normal diet were compared with those fed the high-fructose or high-fat diets. Rather than exacerbating ozone-induced injury or inflammatory effects, both the high-fructose and the high-fat diets dampened some of the toxic responses, which might suggest diminished ability of animals to respond to a stressor.

Cumulative early life environmental and physiological insults impairing ability of an organism to respond to stressors encountered later in life might result in accelerated frailty and aging. More research is needed to understand how diet may affect other biological responses induced by ozone. Our data suggest that there are complex interactions between high-fat or high fructose diets and ozone that need careful assessment. A more comprehensive approach examining effects in multiple organs with high-throughput technologies could provide a better picture of how these diets are likely

to change responses to toxicant exposures. This information will be useful to support epidemiological studies addressing cumulative effects of multiple stressors.

2.5 Beneficial versus detrimental effects of fatty acid dietary supplements on ozone-induced health effects

Another study examined how unsaturated fatty acid supplementation affected ozone-induced pulmonary and metabolic responses. Health benefits and detriments of unsaturated versus saturated fats, respectively, have been well documented (Li et al., 2015; Siri-Tarino et al., 2015). Unsaturated fatty acid supplements enriched in omega-3 and omega-6 are popular because of their antioxidant and anti-inflammatory properties and health benefits for the onset and reduction of major chronic illnesses. These dietary supplements are widely available and heavily consumed by human populations, including those socially disadvantaged (http://www.fao.org/3/a-i7846e.pdf). Based on studies in humans showing beneficial effects of fish oil supplements on increased vessel contractility induced by inhaled particulate matter (Tong et al., 2012), we conducted a systematic study examining pulmonary, cardiovascular, metabolic, and systemic effects of ozone in animals fed diets supplemented with saturated fats (coconut oil), omega-3 fatty acids (fish oil) or omega-9 (olive oil). These studies resulted in four publications (Snow et al., 2018, 2021a; Tong et al., 2020; Valdez et al., 2019) outlining the beneficial and detrimental health effects associated with these dietary supplements, especially when consumed in large quantities by rodents.

A study by Snow and co-investigators (Snow et al., 2018) demonstrated that, in rats (like in humans), the fish oil-enriched diet reduced circulating triglycerides and the vasoconstriction response to inhaled ozone, suggestive of a beneficial health outcome. These findings were concordant with human data linking ameliorative effects of dietary fish oil on health insults evoked by air pollutants (Chen et al., 2022). However, in rodents, this diet also led to accumulation of foam cells in the lung, suggesting suppression of lipid-rich surfactant recycling and associated inflammation. This could have happened because dietary lipids are incorporated into cellular components in the body, and the lung is rich in surfactant, comprised of 80% lipids. Thus, high levels of omega-3 fatty acids could change the surfactant composition or cause oxidation of lipids because of continuous contact of oxygen with surfactant in the lung. This aspect could be related to the high dose of omega-3 used in the study, or omega-3 oxidation causing lipid modifications and accumulation. Snow et al. (2018) observed that lipid transporters and metabolic markers were suppressed, suggesting inhibition of surfactant recycling and accumulation of surfactant in alveolar macrophages leading to foam cell formation (Figure 2.4). Follow-up on these observations is needed to determine how and under what circumstances the omega-3 fatty acid supplements could be causing pulmonary issues. This study raises an important caveat in assessing a limited number of biological functions for determining health benefits of dietary supplements, as not all biological effects might be beneficial. Examining multiple aspects of health effects using animal models is essential in system level understanding of diet and environmental interactions.

Figure 2.4. Ozone induced metabolic, vascular, and pulmonary effects in rats receiving diets enriched with coconut oil, fish oil or olive oil.

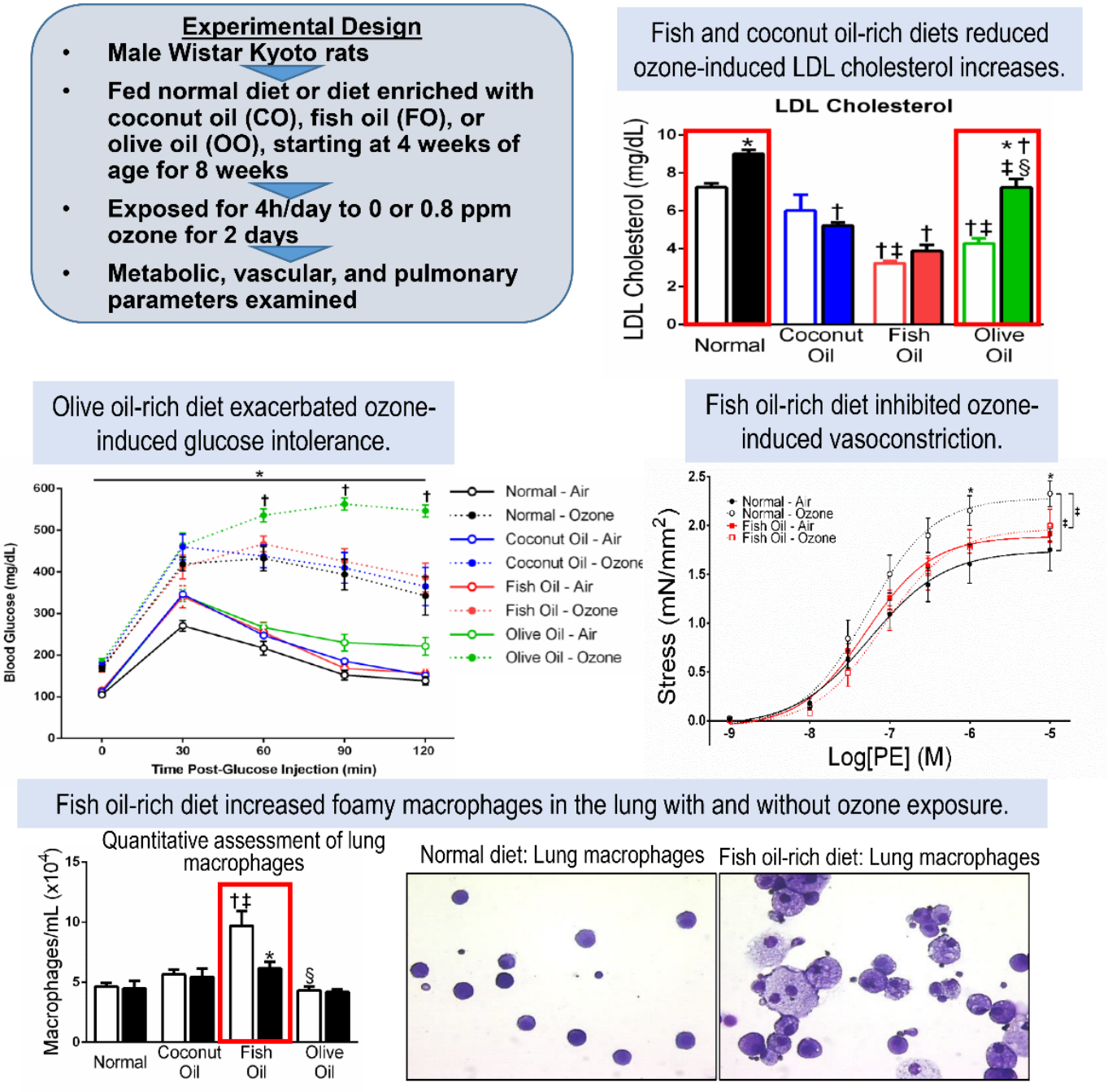


Figure 2.4. Ozone induced metabolic, vascular and pulmonary effects in rats receiving diets enriched with coconut oil (CO), fish oil (FO) or olive oil (OO). These data are from Snow et al. Toxicol Sci. 2018; 163(1):57-69 and Snow et al. Toxicol Appl Pharmacol. 2021a Jan 1;410:115337. For additional details, please refer to these publications.

The second paper from Snow et al. (2021a) examined the metabolic effects of dietary fatty acid supplements after ozone exposure. Ozone exposure occurred at 0.8 ppm for 4 h/day for 2 consecutive days. Results suggested that dietary intake of saturated and unsaturated fatty acids can modify neuroendocrine metabolic responses to inhaled pollutants in multiple tissues. Young healthy rats fed coconut oil or olive oil but not fish oil-enriched diets had increased body fat, liver lipid accumulation, lipidemia, and leptinemia, whereas only animals fed olive oil demonstrated exacerbation of glucose intolerance induced by ozone (Snow et al., 2021a). In addition, these data clearly demonstrated beneficial effects of fish oil supplement in lowering circulating triglycerides. Circulating branched-chain amino acids, an indication of the intensity of the ozone-induced stress response, were increased only in normal diet and coconut oil fed animals after ozone exposure, suggesting dampening of this response by fish oil and olive oil. Diet-specific changes in gene expression were noted in the liver and adipose tissues from air-exposed animals, whereas ozone-induced changes in gene expression in adipose tissue and liver were modified by diet (Snow et al., 2021a). These findings suggest that dietary supplements with unsaturated lipids can change acute homeostatic responses induced by irritant air pollutants in a tissue-specific manner and contribute to variations in susceptibility to metabolic disorders.

In the Valdez et al. (2019) publication leveraged from the same study, the effect of dietary supplements on various brain regions was examined, focusing on mitochondrial function and the neurotransmitters involved in sympathetic responses of the autonomic nervous system. This study demonstrated that unsaturated fatty acid supplementation, specifically fish oil, garnered partial protection for specific brain regions from ozone-induced decreases in mitochondrial respiratory chain enzymes. However, the effects of ozone on mitochondrial bioenergetics are not consistent across enzyme complexes nor across brain regions, suggesting that susceptibility of different brain compartments to ozone is variable. Moreover, this study indicated that diet significantly affected the reactivity of both astrocytes and glia cells in animals exposed to ozone, suggesting that dietary lipids can modify environmental influences on brain, and these changes could affect behavior. Thus, dietary constituents can be considered modifiable risk factors for health outcomes in response to various environmental contaminants.

Tong et al. (2020) examined cardiac impacts of dietary supplements in the same animals used in Valdez et al. (2019) and reported that epigenetic microRNA changes might mediate cardiac effects of ozone, and these effects were attenuated in animals fed a fish oil-supplemented diet. Thus, unsaturated fatty acid dietary supplements may offer protection for some of the physiological effects of environmental stressors; however, caution should be exercised to avoid unwanted side effects. As human dietary habits vary greatly and socioeconomic conditions influence the type of diet being consumed, assessing all interactive influences of diet and environmental conditions on human health is challenging. Nevertheless, health benefits of dietary interventions in individuals exposed to environmental stressors will not only depend on diet itself but also the specific health outcome being assessed.

2.6 Susceptibility to environmental stressors of diabetics consuming unhealthy diet

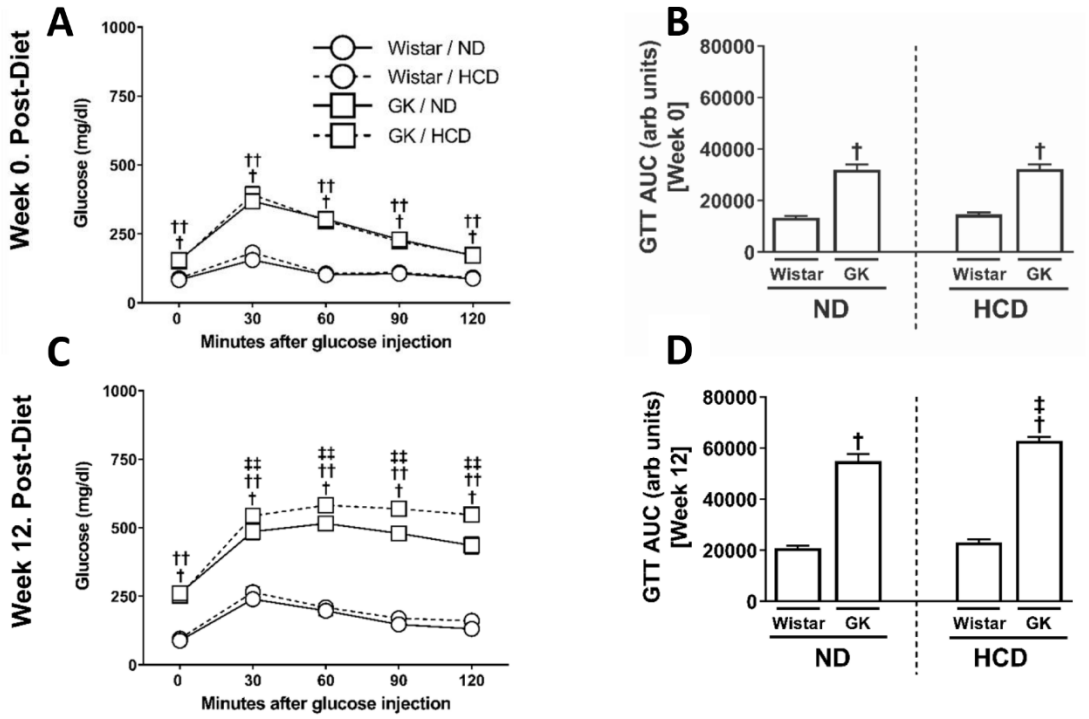
Two related epidemics, diabetes and obesity, represent major public health burdens, both in the United States and globally. According to the World Health Organization (WHO), the incidence of diabetes worldwide increased from 108 million people in 1980 to 422 million people in 2014. Moreover, global incidence of obesity has tripled since 1975, affecting 39% of adults over age 18 in 2016. Obesity and diabetes contribute to cardiovascular disease and collectively drive a constellation

of health effects referred to as metabolic syndrome. Although environmental factors contribute to disease susceptibility, the understanding of how preexisting diseases contribute to exacerbation of environmentally induced health effects is necessary to protect the most vulnerable.

The susceptibility of those with diabetes and consuming an unhealthy diet to environmental stressors, such as ozone, was examined in an animal model of diabetes consuming a high cholesterol diet. Snow et al. (2021b) showed that rats with non-obese diabetes mellitus associated with insulin insufficiency and resistance are more sensitive to high cholesterol diet-induced obesity and glucose intolerance than are healthy rats. Diet containing high cholesterol, however, produced pathological changes in the liver indicative of lipid accumulation when compared with the diabetic rat, which demonstrated inflammatory cell influx. Exposure to ozone further exacerbated glucose intolerance and further increased insulin levels in diabetic rats. Collectively, these results indicated that peripheral metabolic impairment and adiposity could enhance the susceptibility of diabetic individuals to ozone, an inhaled pollutant, and environmental stressors (Figure 2.5).

In another publication, Snow et al. (2021c) demonstrated that diabetic rats have a prolonged, but not necessarily heightened pulmonary injury response to ozone relative to healthy rats. Systemic inflammation, often associated with chronic disease, was induced by a high cholesterol diet and exacerbated by ozone, as indicated by inflammatory markers. Strain-related differences in thrombogenic effects of diet and ozone were noted, consistent with an unhealthy high-cholesterol diet being a risk factor for vascular impairment. Large differences in *ex vivo* vascular contractility were noted between healthy and diabetic rats receiving normal diet, in that diabetic rats were not able to respond to the stressor effect of ozone, but healthy rats were (Figure 2.5). It is important to note that feeding a high-cholesterol diet in healthy animals made them nonresponsive to an ozone stressor effect.

Figure 2.5. Glucose tolerance test (GTT) in healthy Wistar and diabetic Goto Kakizaki (GK) rats during a 12-week dietary regimen with normal or high cholesterol diet.



Normal Wistar but not Diabetic (Goto Kakizaki, GK) rats respond to ozone by increasing vasoconstriction, and when fed high cholesterol diet (HCD), healthy rats behave similar to diabetic rats.

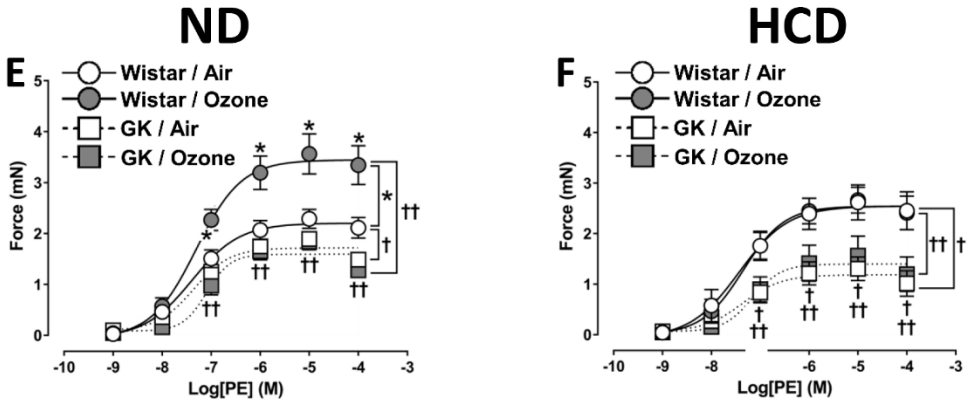


Figure 2.5. A-D: Glucose tolerance test (GTT) in healthy Wistar and diabetic Goto Kakizaki (GK) rats during a 12-week dietary regimen with normal or high cholesterol diet. Values represent mean ± standard error of n=36 animals. Significant strain differences within the same dietary group are shown by "†" and diet-related difference within the same strain by "‡". The respective area under the curve data are shown in the right panel of bar graphs. E-F: Ozone-induced changes in aortic contractility after day 1 in Wistar and GK rats maintained on normal diet (ND) or high-cholesterol diet (HCD). Necropsies were performed within 2 h after air or 1.0 ppm ozone exposure for 6 h. The data show mean ± standard error (n=6/group). * Indicates significant ozone effect ($p \leq 0.05$) in matched strain and diet groups, † indicates significant rat strain effect in matched diet and exposure groups. Abrreviation: PE, phenylephrine. Figures from Snow et al. (Toxicol Appl Pharmacol. 2021b and 2021c)

The study showed a potential mechanistic link between the vasoconstrictive effect of ozone in rats fed a healthy diet and the lack thereof in susceptible animals of a biomarker expressed in blood vessels (Snow et al., 2021c). These data demonstrated that diabetes and a high-cholesterol diet may independently exacerbate specific pulmonary, systemic, and vascular effects of inhaled pollutants. Together, these papers show that healthy and diabetic individuals might be differentially susceptible to high-cholesterol diet and acute air pollutant-induced systemic inflammation and vascular contractility changes. Examination of liver gene expression of markers involved in metabolic processes, such as glucose and lipid metabolism, indicated greater susceptibility of diabetic rats to metabolic impairment induced by ozone exposure (Figure 2.6). These data show that a hepatic steatosis-like phenotype may be induced in diabetic individuals when exposed to ozone.

Collectively, the studies involving the diabetic rat model and a high cholesterol diet interacting with an environmental stressor (ozone) provide significant insights into how preexisting diet-induced systemic inflammation and type 2 nonobese diabetes may independently exacerbate cardiovascular and metabolic disease after exposure to ozone (Snow et al., 2021b,c). The large sector of the population suffering from type 2 diabetes could constitute a subpopulation with increased susceptibility to environmental exposures. This study provides mechanistic evidence to support the exacerbated health risks from environmental exposure in susceptible individuals.

Figure 2.6. Ozone-induced changes in liver mRNA expression in Wistar and GK rats exposed to air or Ozone for 1 day.

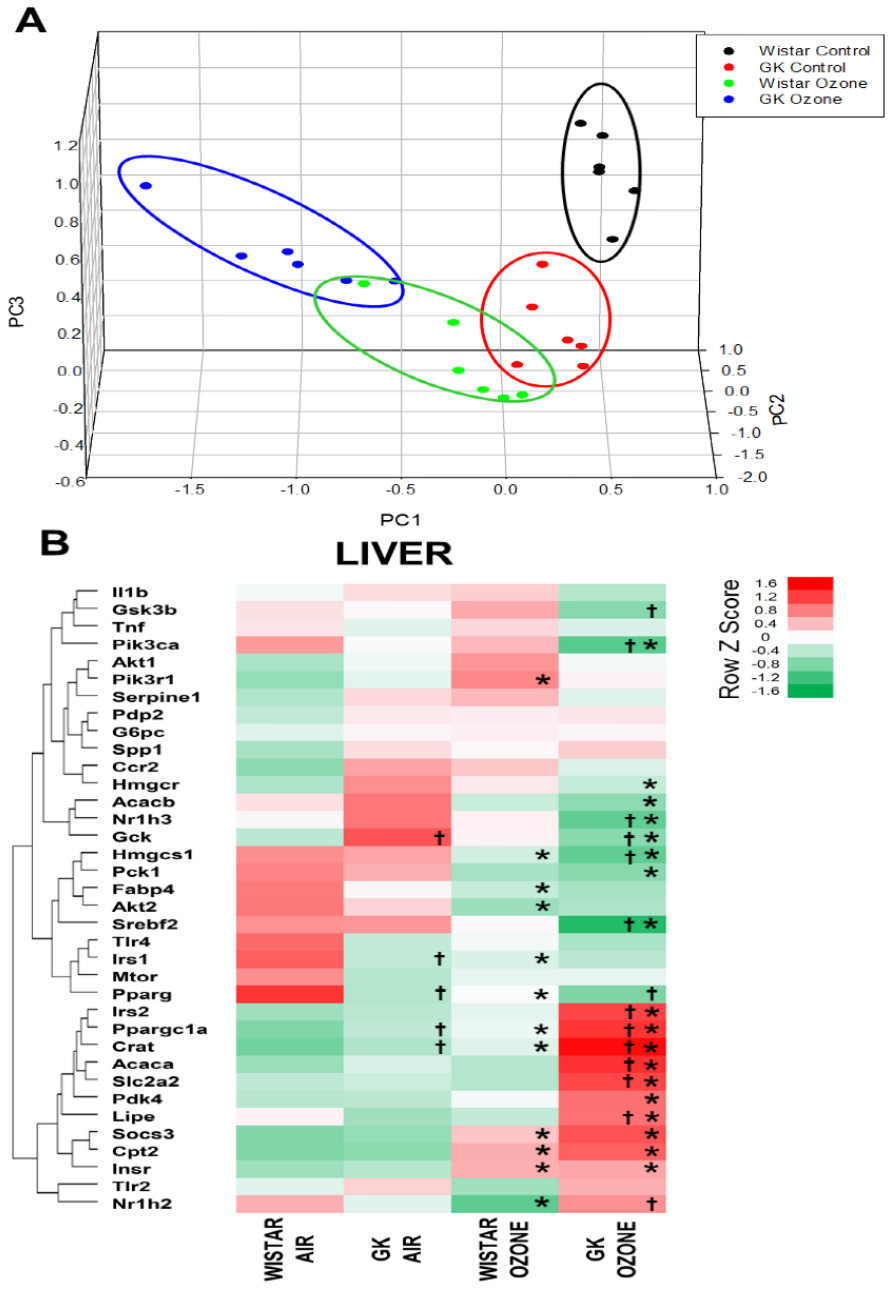


Figure 2.6. Ozone-induced changes in liver mRNA expression in Wistar and GK rats exposed to air or ozone for 1 day. Data are presented as Principal Component Analysis (A) and heat map (B). Gene expression was determined using Illumina mRNA sequencing for the custom panel of (n=6 animals/group). The data were normalized, and raw *z*-scores were calculated. Significant differences were calculated where "*" shows an ozone effect within a given strain and "†" shows a strain effect within a given exposure. Hierarchical clustering was performed using Average Linkage and Euclidean Distance Measurement. From Snow et al. (Toxicol Appl Pharmacol. 2021c).

2.7 Studies examining chemical and nonchemical stressor interactions, pregnancy outcomes, and developmental impact

The ”Developmental Origins of Health and Disease” hypothesis proposes that exposure to environmental influences during critical periods of development and growth may have significant consequences on an individual’s life-long health and disease risk. Maternal obesity has been shown to be one of these influences. Children born to obese mothers have been shown to be more susceptible to allergic inflammatory conditions and suffer a higher incidence of asthma. Gordon et al. (2017b) and Snow et al. (2019) showed that a high-fat diet (given 1 month before mating and maintained throughout gestation and lactation) significantly and persistently increased maternal body weight and body fat during the entire course of pregnancy in Long-Evans rats (Figure 2.7). Despite cessation of the high-fat diet at 35 days of age, elevated body weight and increased body fat of offspring persisted throughout the study.

Figure 2.7. Body weight of Brown Norway (BN) rat offspring whose mothers were fed control or high-fat diet starting at 30 days of age.

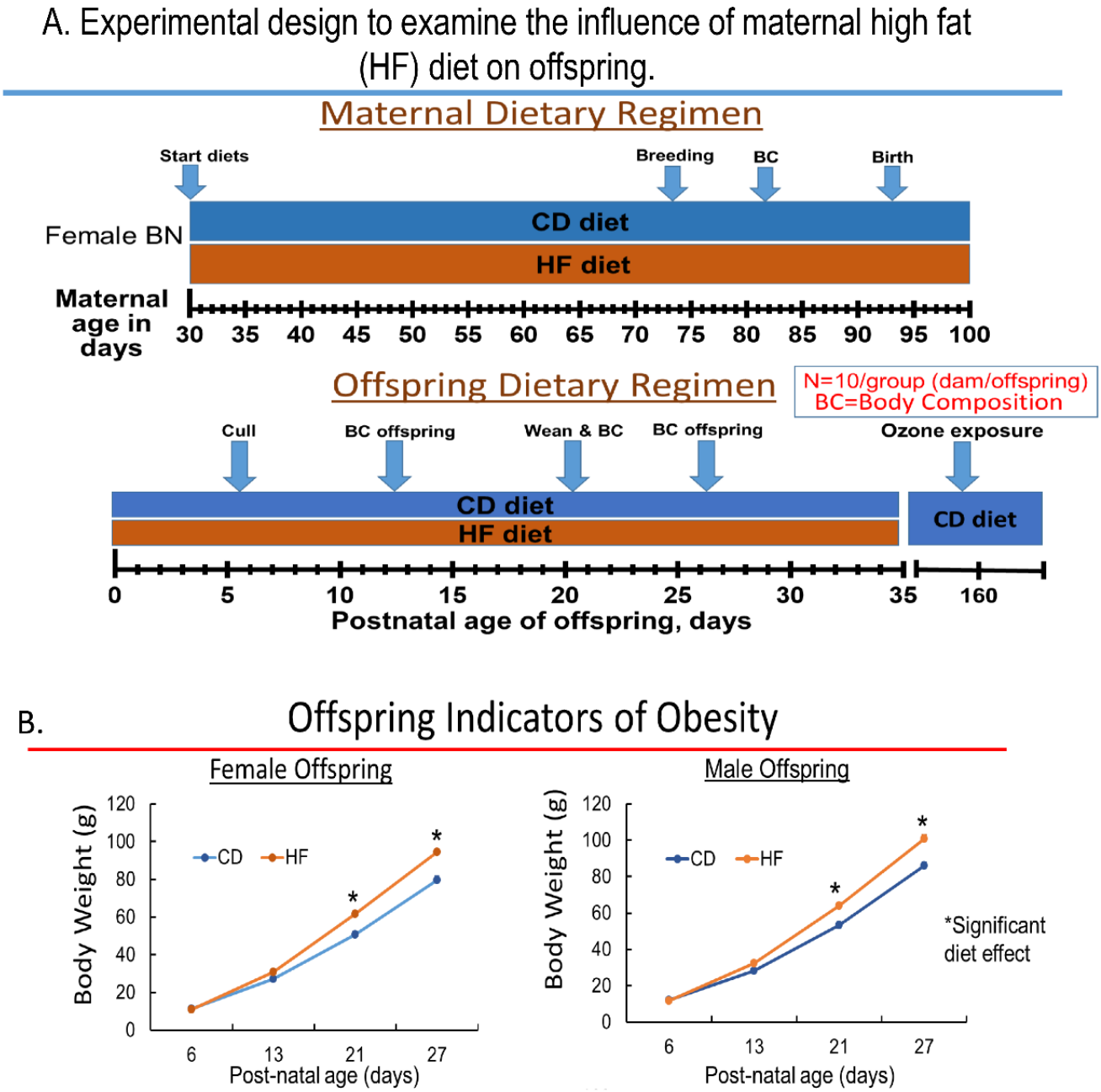


Figure 2.7. A. Brown Norway (BN) females were fed control (CD) or high-fat (HF) diets starting at age of 30 days. Breeding occurred at 72 to 74 days of age, and then offspring were weaned at 21 days and maintained on same diets until age of 35 days. B. Offspring body weight and body fat composition. Data from Gordon et al. (Inhal Toxicol. 2017;29(6):239-254) and Snow et al. (J Toxicol Environ Health A. 2019;82(2):86-98.

At 90 days of age, offspring were exposed to ozone for 2 consecutive days. Ozone-induced alterations in pulmonary injury (Figure 2.8) and inflammation (Figure 2.9) were exacerbated significantly by maternal high-fat diet and resultant offspring adiposity. These findings suggest that maternal high-fat diet may enhance the susceptibility of offspring to the adverse health effects of air pollutants.

Figure 2.8. Ozone-induced pulmonary vascular leakage is exacerbated by maternal and postnatal high fat diet in male BN offspring.

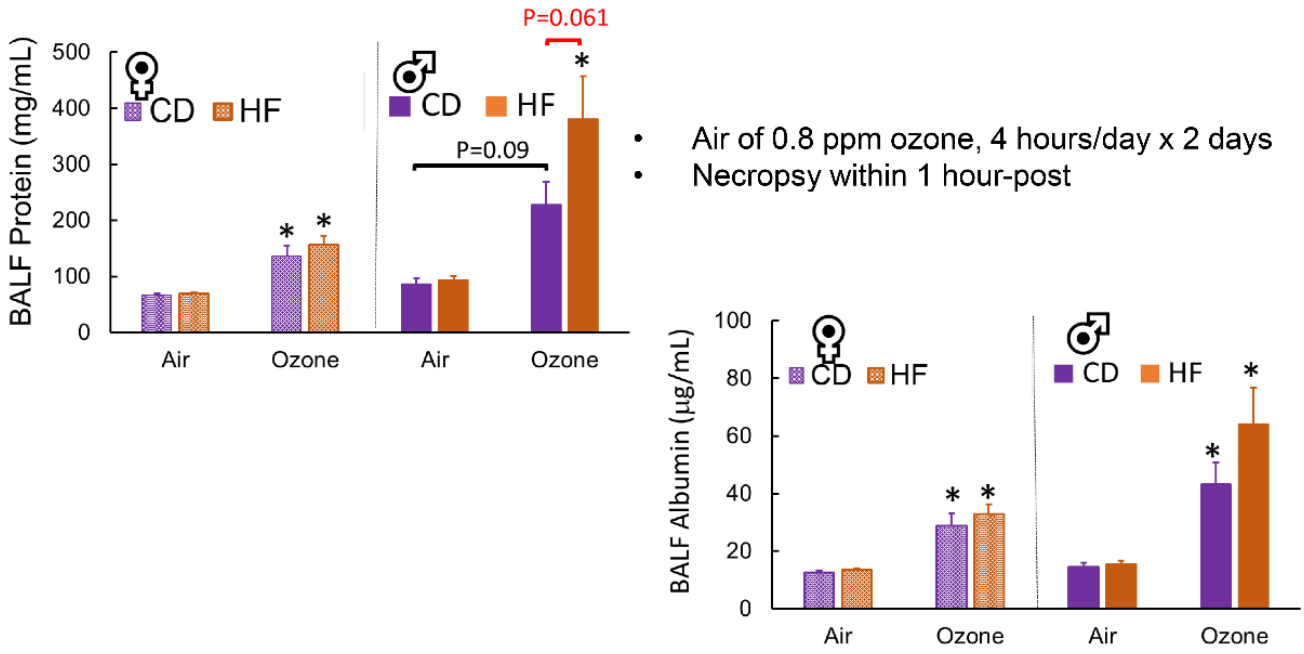


Figure 2.8. Ozone-induced lung injury and inflammation in male and female offspring from mothers fed control or high-fat diet. Brown Norway (BN) females were fed control (CD) or high-fat (HF) diets starting at 30 days of age. Breeding occurred at 72 to 74 days of age, and then offspring were weaned at 21 days and maintained on same diets until age of 35 days. Then they were exposed to air or 0.8-ppm ozone (4 h/day x 2 days) followed by assessment of biomarkers. Data from Gordon et al. (Inhal Toxicol. 2017;29(6):239-254) and Snow et al. (J Toxicol Environ Health A. 2019;82(2):86-98).

Figure 2.9. Ozone-induced neutrophilic inflammation is exacerbated by maternal and postnatal high fat diet in male BN offspring.

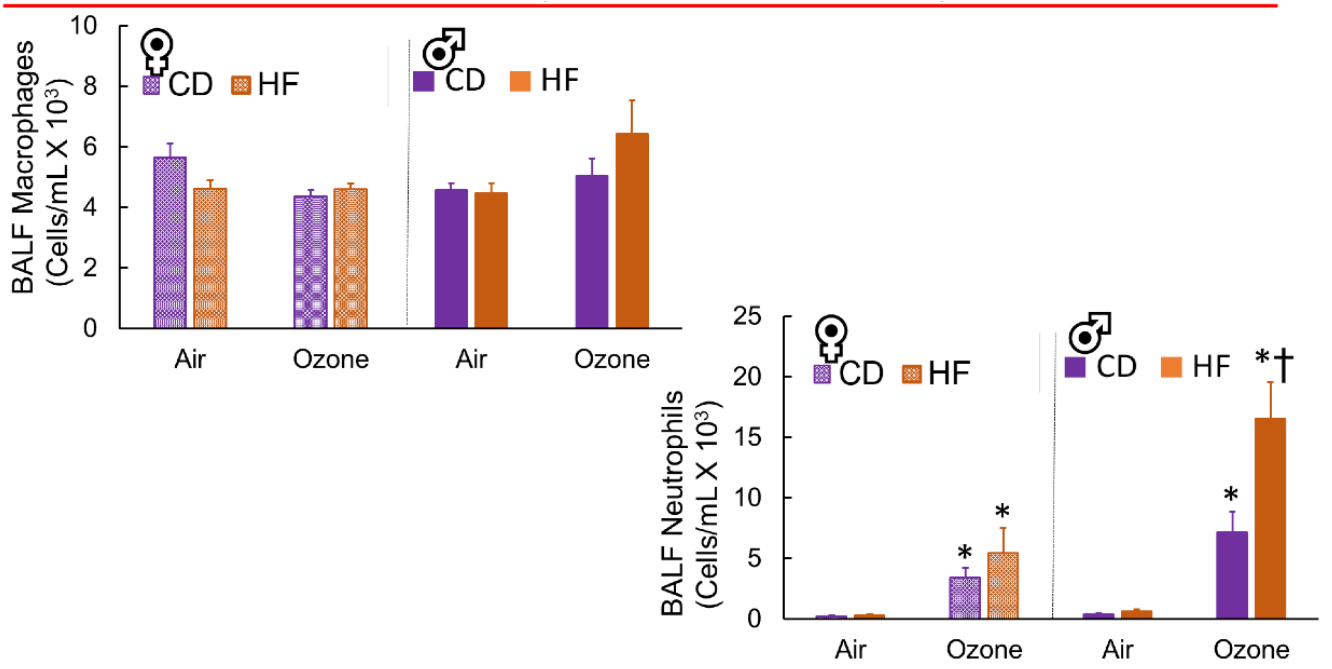


Figure 2.9. Ozone-induced lung inflammation in male and female offspring from mothers fed control or high-fat diet. Brown Norway (BN) females were fed control (CD) or high-fat (HF) diets at age of 30 days. Breeding occurred at 72 to 74 days of age, and then offspring were weaned at 21 days and maintained on same diets until age of 35 days. Then they were exposed to air or 0.8-ppm ozone (4 h/day x 2 days), followed by assessment of biomarkers. Data from Gordon et al. (Inhal Toxicol. 2017;29(6):239-254) and Snow et al. (J Toxicol Environ Health A. 2019;82(2):86-98).

Miller et al. (2017) reported that ozone exposure of pregnant rats during implantation on gestational days (GDs) 5 and 6 led to fetal growth restriction likely associated with diminished maternal uterine arterial blood flow during late gestation (GD 15 to 21). Miller and collaborators further examined the effects of ozone on the implantation process and described a reduction of serum cytokines known to promote implantation. Additionally, an *in vitro* study with human first trimester, placental-derived trophoblast cells (that are involved in implantation) showed that ozone down-regulated metabolic capacity, wound closure, and invasion but increased release of a critical inhibitor of invasion and angiogenesis (Miller et al., 2019a). To confirm these ozone effects on implantation, low-dose aspirin (known to lower the risk of preeclampsia and intrauterine growth restriction in high-risk pregnancies clinically) was given to rats during early pregnancy (GD 1 to 7) along with ozone exposure (GD 5 and 6) (Miller et al., 2019b). Aspirin treatment produced marginal improvements in ozone-induced uterine blood flow in the pregnant rats and was effective in mitigating the fetal growth deficits associated with this air pollutant. Aspirin treatment for the entire course of pregnancy increased placental weight and reduced its antioxidant status, indicative of placental insufficiency. Hence, the protective effects of aspirin were specific to the exposure window of ozone during implantation. A more recent study examined how sex-specific placental adaptation may impact fetal growth and metabolic phenotype when ozone exposure occurs during implantation (Miller et al., 2020). Taken together, these findings indicate that ozone exposure during pregnancy may impair fetal growth through interruption of the

implantation process, a reduction of angiogenesis, reduced maternal blood flow and supplies of nutrients to the fetus during late gestation. In a companion study, Miller and collaborators also reported on how exposure to ozone and noise during pregnancy may alter fetal growth (Miller et al., 2019c). This study showed ozone exposure and noise differentially impacted uterine blood flow, particularly at mid-gestation, with only ozone exposure causing sex-dependent fetal growth restriction in male offspring. Maternal stressors during pregnancy in communities with environmental justice issues and the impact on child developmental processes are important health concerns for EPA. These data suggest that stress during implantation could result in impaired pregnancy outcomes.

2.8 Maternal high-fat diet and impact on offspring metabolism and gut microbiota

Maternal exposure to endocrine-disrupting chemicals, psychosocial stressors, and steroids during pregnancy have been postulated to interactively alter fetal hypothalamic programming of metabolic homeostasis and increase the offspring's susceptibility to metabolic diseases. Snow and collaborators (2020b) conducted research to examine whether maternal obesity and high-fat diet altered ozone-induced hormonal and metabolic changes in peri-adolescent male and female offspring using clinical and hormonal assessments and global serum metabolomics (Figure 2.10).

This study (Snow et al., 2020b) demonstrated that maternal high-fat diet and obesity resulted in a small degree of obesity in offspring, with changes in circulating lipid profiles and benzoate metabolites, suggesting changes in gut microbiota in both male and female offspring. When exposed to ozone, these offspring experienced heightened metabolic changes. Female offspring were more affected than males. Some ozone-induced changes in metabolic processes were suggestive of mediation through neuroendocrine pathways involving stress hormones. Collectively, these data showed that maternal obesity and high-fat diet predisposed offspring to metabolic alterations reflective of impaired mitochondrial function, altered lipid and protein metabolism, and changes in gut microbiome when challenged with an environmental stressor. An example of ozone-induced changes in circulating biomarkers of the gut microbiome in the offspring from dams fed a high-fat diet is shown in Figure 2.10.

These data provide insight into how maternal high-fat diet might result in greater susceptibility of female offspring to metabolic disorders through complex interactions between key metabolic pathways when challenged with environmental stressors. This is distinct from increased susceptibility of male offspring to ozone-induced lung injury and inflammation (Gordon et al., 2017). These outcomes imply that differential responses to environmental stressors might be due to the type of response being assessed; and emphasizes the importance of a systems approach at the organismal level and the limitations of the use of in vitro approaches. These data provide mechanistic understanding and support health assessment involving early life stressor exposure during development and susceptibility to metabolic and immune disorders later in life.

Figure 2.10. Ozone-induced changes in the blood metabolites of male and female offspring born to obese mothers on high-fat diet.

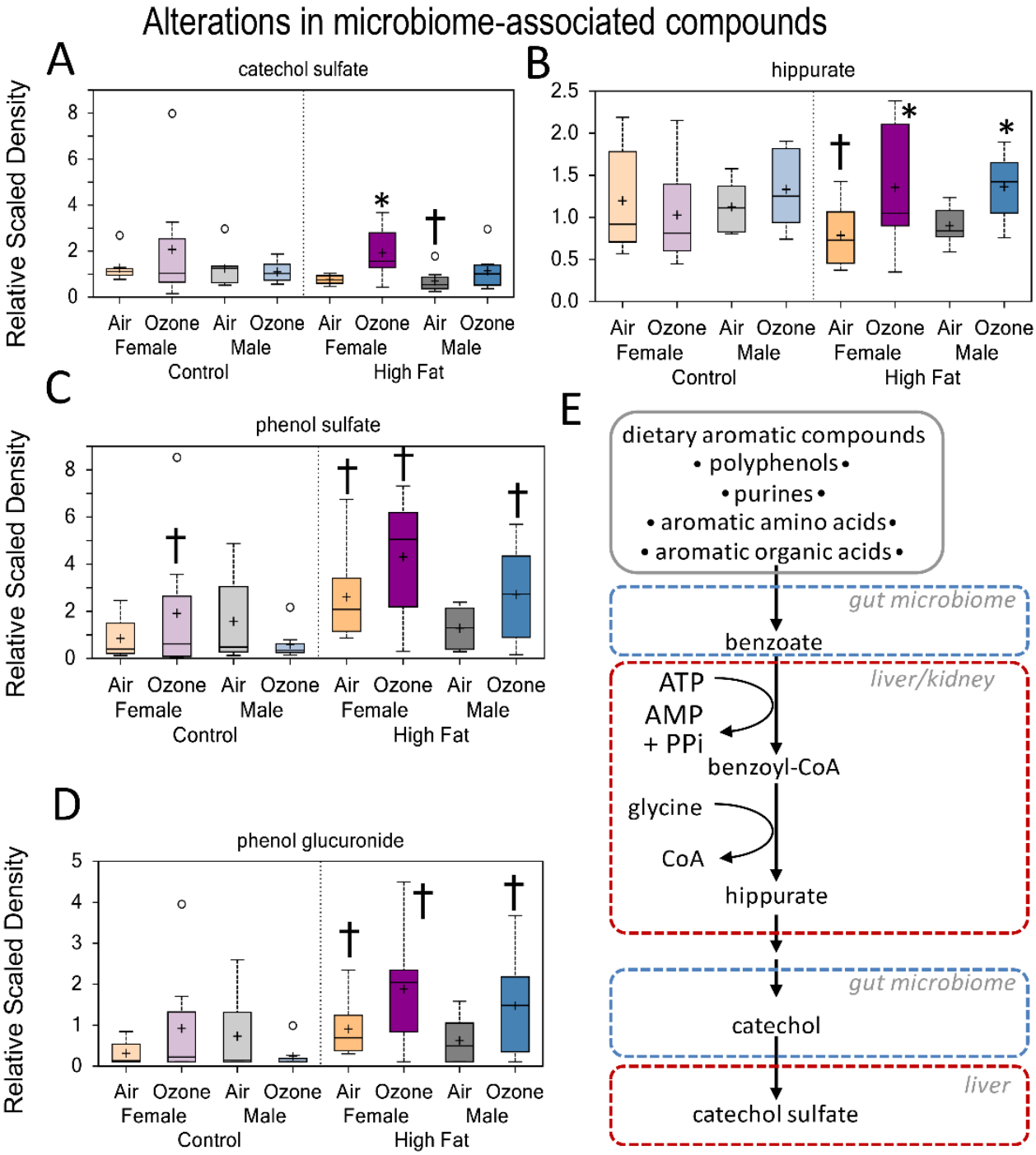


Figure 2.10. Ozone-induced changes in the blood metabolites of male and female offspring born to obese mothers on high-fat diet. Global metabolomic analysis of serum samples identified specific metabolite changes linked to gut microbiome in offspring because of maternal high-fat diet and ozone exposure. A through D show individual metabolites. E shows metabolic pathway for gut microbiome-induced changes that result in production of metabolites. From Snow et al., Scientific Reports. 10(1):16353. PMID: 33004997, 2020b.

2.9 Conclusions and future perspectives

EPA's mission includes protection of health from environmental stressors for the most vulnerable and susceptible individuals. Compromised health and unhealthy lifestyle can interactively exceed a tipping point when additional environmental stressors are encountered. A current and striking example is the susceptibility of socially disadvantaged, frail, old, and unhealthy individuals to COVID-19 infection and mortality. Understanding multiple risk factors can elucidate biological mechanisms, which is critical for making health-related decisions and developing mitigation strategies. Our studies show that risk factors, such as age, sedentary lifestyle, and unhealthy diet, can exacerbate health effects of ozone exposure in a health-outcome-specific manner. For example, sedentary animals had greater glucose intolerance after ozone exposure than those exercising; although having a smaller effect on respiratory function, older animals have a delayed recovery after ozone exposure, and high fructose diet is associated with a blunted ozone-induced inflammatory response. Likewise, maternal high-fat diet was associated with changes in markers of insulin signaling and gut microbiome alterations in both male and female offspring, and ozone exposure led to exacerbation of these effects in female offspring while also affecting mitochondrial respiration. A fish oil-supplemented diet protected rats from vascular effects of ozone but produced lung inflammation and accumulation of foamy macrophages, whereas olive-oil-rich diet exacerbated ozone-induced glucose intolerance. Atherogenic diet is associated with impaired vascular response to ozone and systemic inflammation. These findings suggest diet and stressor-specific interactions can lead directly or indirectly to exacerbation of health effects of ozone exposure. Continued research in understanding the underlying mechanisms of these interactions will provide better mitigation strategies and inform decision making.

2.10 References

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Section 3. Perinatal exposure to manganese and psychosocial stress

3.1 Introduction

There is ample evidence suggesting that early life experiences can have profound impacts on the life course of individuals, potentially increasing susceptibility to various health risks throughout their lifetimes (Hackman et al., 2010; Pollanska et al., 2012; Gaillard et al., 2019). The extent of these early life experiences is vast, but most are related directly to the physiological and psychological well-being of the expecting and nursing mother, as well as her environmental circumstances. These influences can impact multiple organ systems and physiological processes, but the developing brain is particularly vulnerable to disruption, as structural and functional reprogramming are difficult to reverse, which can result in increased risk of physical and mental health damage (Bellis et al., 2019; Hertzman, 1999; Hughes et al., 2017)

From an environmental justice perspective, adverse life experiences occur within the context of one's socioeconomic status (SES). Particularly vulnerable are those living in communities of lower SES because of multiple stressors likely encountered (e.g., deteriorated living conditions, economic disparity, limited access to social resources and community support, lack of healthy diet, and increased exposure to environmental pollution). Several human studies have shown a connection between stress experienced by a mother and an increased risk that her child will develop a range of adverse cognitive and behavioral deficits, including anxiety and depression (Heim and Nemeroff, 1999; Hackman et al., 2010; Polanska et al., 2012; Gee and Casey, 2015; Ruiz et al., 2016; Nilsen and Tulve, 2020). These epidemiological findings largely have been confirmed by well-controlled laboratory animal studies (Campos et al., 2013; Lehmann et al., 2000; Weinstock, 2008; 2017; Markham et al., 2010; Schultz et al., 2011; Wilson et al., 2012). Both human and animal studies have implicated sustained increases of maternal stress hormones (i.e., cortisol for humans or corticosterone for rodents) during gestation as a key indicator of adverse health outcomes in offspring (Weinstock, 2005; 2008). A pattern of sustained hormonal response is different from the acute "flight-or-fight" stress response that wanes when the impending danger disappears. Maternal glucocorticoids can reach the fetal brain through placental transfer and affect the structure and function of the limbic system and the hypothalamic-pituitary-adrenal (HPA) axis (Kapoor et al., 2006; Darnaudery and Maccari, 2008; Grace et al., 2011), resulting in permanent changes due to delayed nervous system development and inhibition of neurogenesis in parts of the brain, in addition to development of immunosuppression, cardiovascular disease, and depression later in life (Jafari et al., 2017; Kapoor et al., 2006).

The disproportionate levels of stress associated with communities of lower SES have drawn considerable attention in discussions about modulation of the health risks of higher exposure to environmental pollutants. For instance, lead is typically found in older houses in urban neighborhoods, levels of volatile organic compounds and metals are high in proximity to highways and industrial facilities, and exposure to pesticides is elevated in migrant farm worker communities. Many of these chemicals are potential neurodevelopmental toxicants and may have lasting and devastating impacts on children's mental health, IQ, and overall cognitive function (Schettler, 2001; Polanska et al., 2012; Grandjean and Landrigan, 2014; Bellinger, 2008).

3.2 Research objectives and approaches

This research used an animal model to explore the interactions between chemical and nonchemical stressors during early life development and focuses on exposure to manganese (Mn) and maternal psychosocial stress. Mn is an essential trace metal that is necessary for normal development and maintenance of nerve and immune cell functions, among many other physiological functions (Keen et al., 2000). However, at higher levels, Mn is characterized as a neurotoxicant (Crossgrove and Zheng, 2004; O'Neal and Zheng, 2015; Keen et al., 2000). Exposure to Mn may arise from its use in the manufacture of iron and steel alloys, from use in the agricultural industry in fertilizers and fungicides, and from its use in gasoline, in the form of methylcyclopentadienyl manganese tricarbonyl (ATSDR, 2012; Davis, 1998; Smith et al., 2018). Diet and water are the primary sources of exposure for the general human population (ATSDR, 2012). Food rich in Mn include grains, fruits, green vegetables, nuts, and teas, as well as dietary supplements. The percentage absorption of Mn varies considerably depending on factors including age, gender, and other diet constituents (ATSDR, 2012; Oulhote et al., 2014b). Health-based screening levels have established a lifetime exposure to Mn in drinking water at 0.3 mg/L (U.S. EPA, 2004). Elevated Mn in drinking water is a concern for many countries, including the United States, where groundwater studies have reported Mn concentrations exceeding the health benchmark in approximately 6.9% of samples (WHO, 2011; McMahon et al., 2019; Mitchell et al., 2011; Wasserman et al., 2006).

Manganese can cross the placenta to reach the fetus and can be transferred to the neonate through mother's milk, leading to deposition in the developing brain (Fechter, 1999; Dorman et al., 2005a,b). Epidemiological studies evaluating the developmental impact of Mn overexposure in humans show associations with hyperactivity, lower intellectual function, impaired motor skills, and attentional impairments in children (Bouchard et al., 2011; Takser et al., 2003; Zoni and Lucchini, 2013; Oulhote et al., 2014a; Grandjean and Landrigan, 2014; Shih et al., 2018). Animal studies have shown similar effects from Mn overexposure during early life. In rodents, Mn exposure during the perinatal period has been shown to reduce motor coordination, increase spontaneous activity and behavioral reactivity (Cordova et al., 2012; Peres et al., 2015; Beaudin et al., 2013; Chandra et al., 1983) cause bi-directional effects on anxiety and hyperactivity (Beaudin et al., 2013; Betharia and Maher, 2012; Amos-Kroohs et al., 2015; Sprowles et al., 2018) impair spatial learning, egocentric learning, and latent inhibition (Molina et al., 2011; Liang et al., 2015; Amos-Kroohs et al., 2017; Sprowles et al. 2018) reduce short-term object and social memory (Peres et al., 2015; Beaudin et al., 2013; Amos-Kroohs et al., 2016) and impair attention (Beaudin et al., 2017). A few of these studies also have investigated interactions of postnatal Mn exposure and postnatal stress on these outcomes and have observed some interactions (Vorhees et al., 2014; Amos-Kroohs et al., 2016; Sprowles et al., 2018).

Our objective was to examine effects of repeated *mild stress* to simulate those experienced in humans living under socioeconomic and environmental hardships. For this study, the gestation and lactation periods were selected for both the chemical and stress exposures because they represent critical phases of neurodevelopment (Rice and Barone, 2000). We started the perinatal stress paradigm on gestation day (GD) 13 and continued until the morning of postnatal day (PND) 9 for half of the pregnant rats. This critical neurodevelopmental plasticity phase in rats approximates the second and third trimesters in human pregnancy and encompasses the development of the hypothalamus (part of the HPA axis that regulates the body's response and adaptation to stressors), hippocampus, and

cerebral cortex that mediate executive cognitive functions, including spatial learning, memory, attention, and impulsivity (Rice and Barone, 2000; Dubovicky et al., 2008).

3.3 Maternal and perinatal stress exposure

The use of animal models to evaluate responses to repeated low-level stress poses technical challenges because rodents are adept at habituating to recurring stressors. Therefore, we employed a series of variable, unpredictable, and noninvasive stressors to produce stress in rats during the perinatal period similar to those employed in other studies (Fride and Weinstock, 1984; Weinstock, 2008; Smith 2012). The stressors were applied in an alternating manner, such that the same stressor did not occur every day or back-to-back, to reduce habituation. During the prenatal period, the stressors consisted of (1) restricted movement in a well-ventilated Perspex® box (7cm wide x 7 cm high x 18 cm deep; the width of area was adjusted for larger pregnant rats) for 1 h; (2) intermittent intervals of filtered white noise (5- to 15-min bursts separated by 50 to 160 min each) in the home cage (85 dB SPL; re: 20 µPa); (3) intermittent intervals of light during the dark cycle (overhead lights on for periods of 15 min separated by 15 to 195 min); (4) overhead lights on continuously for 24 h; (5) 1 h of fox urine odor (trimethylthiazoline ≥ 90%); a few drops on gauze in an open container placed in animal room near the animal rack) with rats in their home cage; and, (6) small housing conditions (new cage size: 7.5 in wide x 11.5 in deep) with reduced bedding for 24 h. Postnatal stress was applied to both stress-group dams and their litters beginning on PND2. Dams and their litters were placed in an altered cage environment comprised of metal grid floors with reduced bedding and nesting material and were undisturbed until the morning of PND9. The schedule of all stress procedures is described in Table 3.1. Doses of Mn were chosen based on published data (Molina et al., 2011; Betharia and Maher 2012; Pappas et al., 1997), as well as concentrations found in regions with contaminated water (U.S. EPA, 2004). Mn was delivered from GD7 to PND22 to pregnant and nursing rats in their drinking water at either 0, 2, or 4 mg Mn/mL water to simulate the most likely route of exposure for pregnant women. This design yielded six treatment groups with Mn dosage-perinatal stress (PS) or no stress (NS) groups: 0-PS, 0-NS, 2-PS, 2-NS, 4-PS, and 4-NS.

Table 3.1. Schedule of perinatal stress exposures

GD13	Intermittent intervals of white noise during light cycle
GD14	1 h of restricted movement in morning and afternoon; Lights on throughout dark cycle
GD15	Placed in small-sized cage with reduced bedding for 24 h
GD16	Returned to regular-sized cage; Exposed to predator (fox) urine odor for 1 h; Lights on for intermittent intervals during dark cycle
GD17	1 h of restricted movement in morning and afternoon; Intermittent intervals of white noise during dark cycle
GD18	Placed in small-sized cage with reduced bedding for 24 h
GD19	Returned to regular-sized cage; Exposed to predator (fox) urine odor for 1 h; Lights on for intermittent intervals during dark cycle
GD20	Placed in small-sized cage with reduced bedding for 24 h
GD21	Returned to regular-sized cage
PND0	Dams undisturbed and allowed to give birth
PND2-9	Dams and pups moved to cages with metal grid floor and reduced bedding (undisturbed except to weigh dams and change bottles biweekly)

Serum corticosterone (CORT) was used as a biomarker indicating the efficacy of the stressors to increase maternal stress. Whole blood samples were collected from the tail vein of the dams prior to the start of any treatment (GD7) and at two subsequent time points (GD16 and PND9). As shown in Figure 3.1, basal serum corticosterone levels were low prior to introducing the stress paradigm/Mn exposure (GD7) and were similar in all groups. Maternal serum CORT levels were higher in the perinatal-stress groups compared with the no-stress groups on GD16, but this altered stress response was attenuated by PND9. Although the CORT levels appeared to be elevated in a Mn-dose-dependent manner (GD16), there was no interaction of Mn and stress in elevating CORT at any time point.

Figure 3.1. Stress treatments increased maternal serum corticosterone levels during pregnancy.

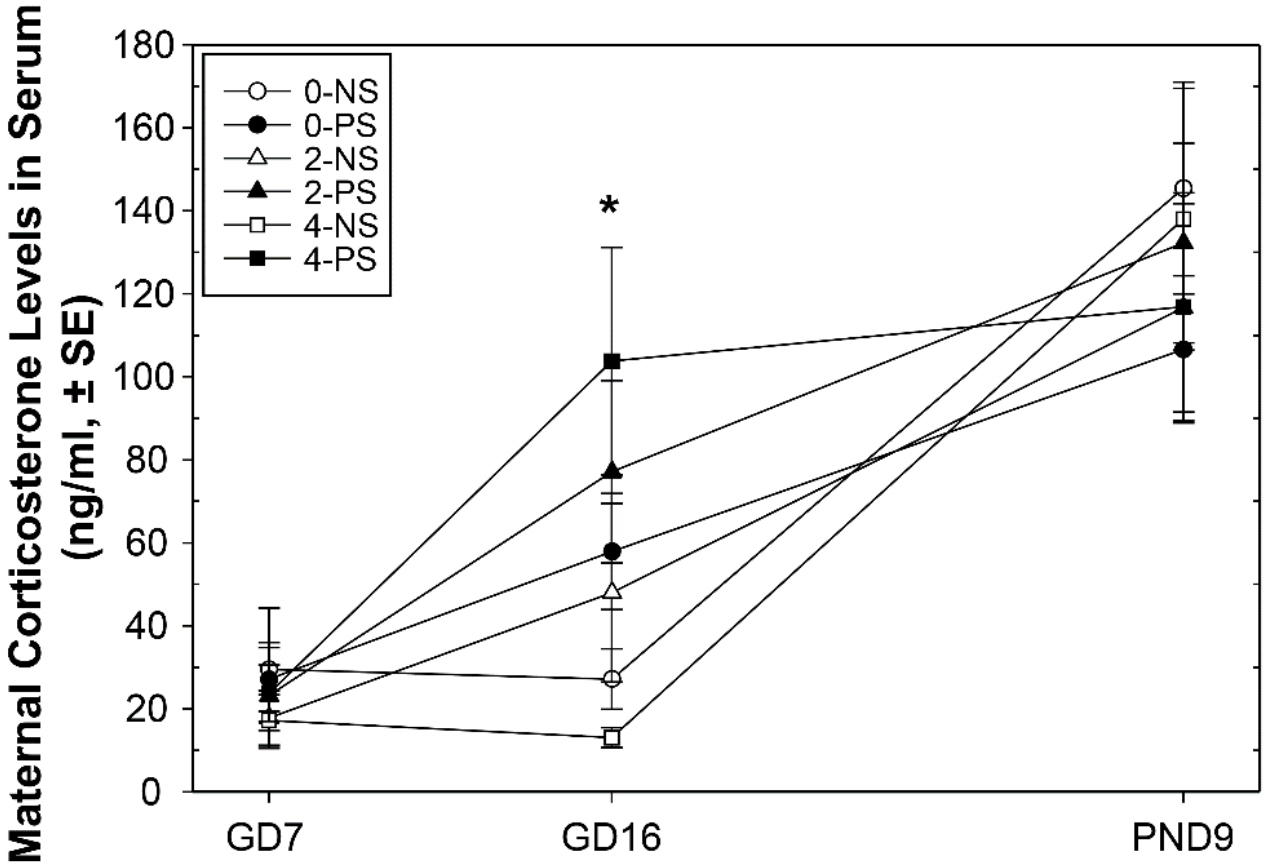

Figure 3.1. Maternal serum corticosterone levels were higher in the perinatal-stress groups compared with the no-stress groups on GD16 (denoted by *). Data are presented as means ± SE. See Beasley et al., *Neurotoxicol. Teratol*. 90:107061, 2022 for details.

3.4 Maternal exposure to manganese in drinking water

No evidence of overt maternal toxicity or obvious differences in maternal care were observed. Body weights of all dams steadily increased throughout gestation. However, there was an effect of both Mn and the perinatal stress paradigm on maternal body weight gain during gestation (Figure 3.2 A). The 0 and 2 mg/mL Mn-exposed groups gained more weight on GD10 and GD21 when compared with the 4 mg/mL Mn group, whereas on GD17 the 0 mg/mL Mn group had gained more weight than the 4 mg/mL Mn group. Further analysis indicated the no-stress groups gained more weight than the perinatal-stress groups on GD17 and GD21 (Figure 3.2 A). Figures 3.2 B, C, and D show the effects of Mn on maternal weight gain. No effects of Mn or stress on maternal weight were observed postnatally.

Figure 3.2. Treatment with manganese (Mn) in drinking water or maternal stress altered maternal weight gain during pregnancy.

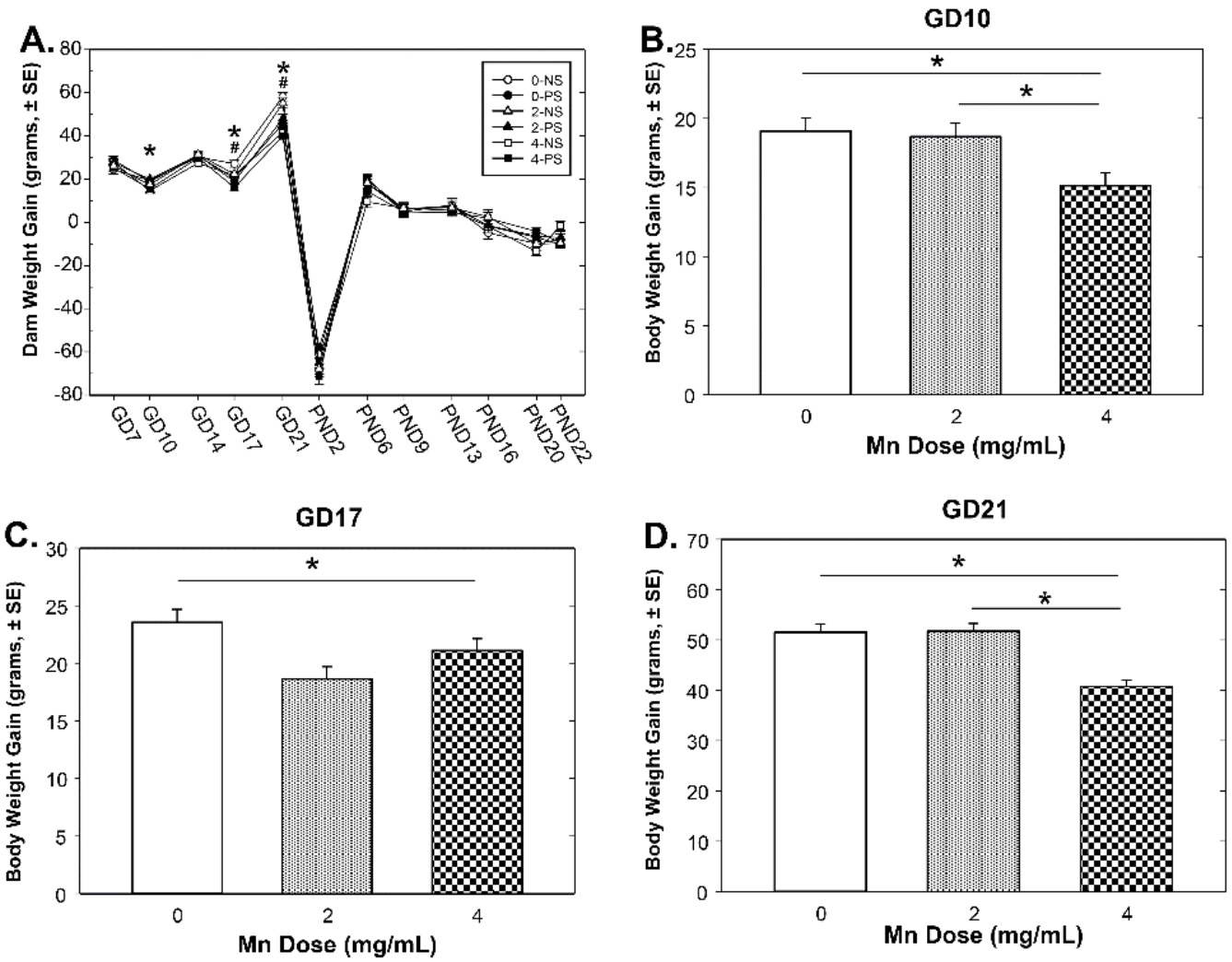

Figure 3.2 A-D. Maternal body weight gain: GD7 through weaning on PND22. Mn dosing began on GD7, and the stress paradigm began on GD13. A: Effects of both Mn (denoted by *) and the perinatal stress paradigm (denoted by #) on maternal body weight gain during gestation. B-D: Bar graphs show the effects of Mn on maternal weight gain from GD7 through the indicated day (differences denoted by *). Data are presented as means ± SE. See Beasley et al., Neurotoxicol. Teratol. 90:107061, 2022 for details.

Daily maternal fluid intake increased across gestation and lactation. However, the overall fluid consumption normalized by body weight was reduced in a dose-dependent manner in the Mn-exposed groups when compared with controls (Figure 3.3). Consumption was lower in the 4 mg/mL Mn-exposed dams on GD10 through PND6 and on PND13 compared with the 0 and 2 mg/mL Mn-exposed groups (possibly indicating the upper limit of palatability of exposure to Mn through drinking water in rats), but, on PND9, there was only a difference between the 0 and 4 mg/mL Mn groups. No effect of stress was observed on water consumption.

Figure 3.3. Maternal exposure to Mn in drinking water or maternal stress altered water consumption during pregnancy.

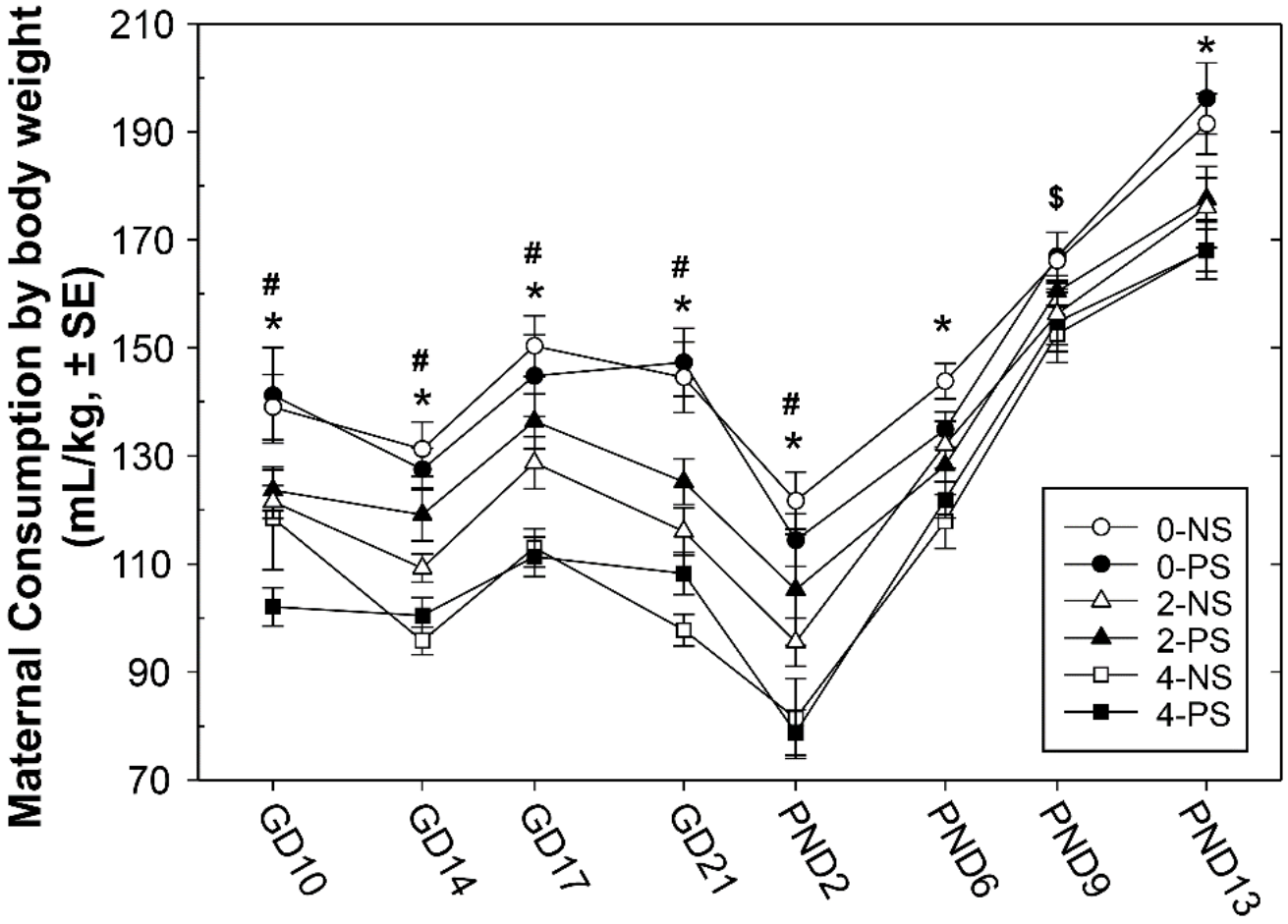

Figure 3.3. Maternal water consumption by body weight (mL/kg). All Mn-exposed groups are different from each other on GD10 through PND2 (denoted by #); the 4 mg/mL Mn group also consumed less than the 0 and 2 mg/mL Mn groups on PND6 and 13 (denoted by *), whereas, on PND9, the 4 mg/mL Mn group only consumed less than the controls (denoted by $). Data are presented as means ± SE. See Beasley et al., *Neurotoxicol. Teratol*. 90:107061, 2022 for details.

There were no treatment-related differences in litter size, male:female ratio, or viability. All litters, regardless of dose or stress level, delivered an average of 4 to 6 male and female pups each. Postnatal weights prior to weaning are litter averages of males and females; after weaning, offspring were weighed individually. Lower weight gain observed in rat offspring in the 4 mg/mL group prior to weaning persisted into young adulthood, but there was no effect of perinatal stress on postnatal growth (Figure 3.4).

Figure 3.4. Maternal treatment with 4 mg/mL Mn in drinking water decreased the body weights of offspring.

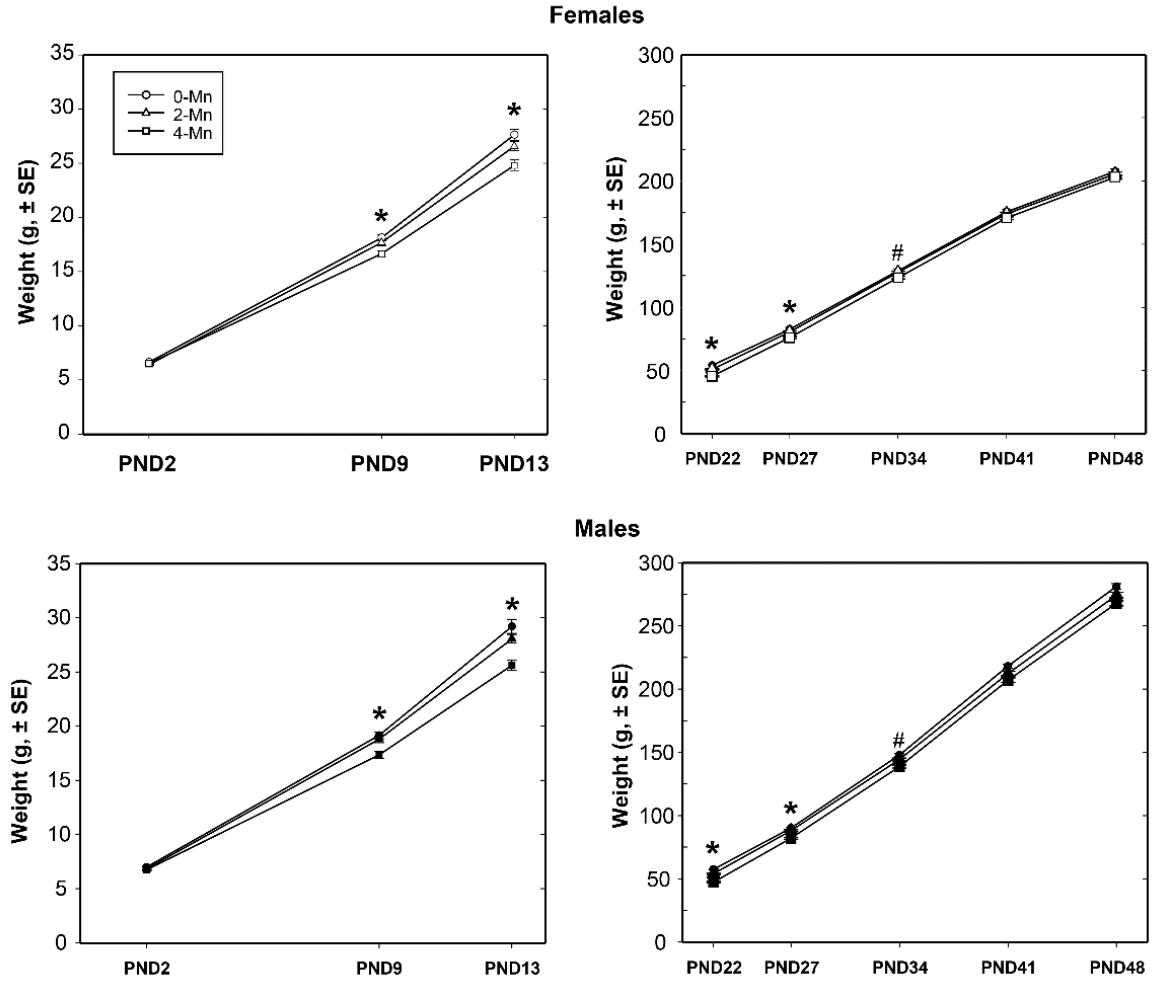

Figure 3.4. Pre- and postweaning body weights of offspring. On PND9 through PND27, the 4 mg/mL Mn-exposed group had lower body weights than the other Mn treatment groups (denoted by *), whereas, on PND34, the 4 mg/mL Mn group was lower than the controls (denoted by #). Data are presented as means ± SE. See Beasley et al., *Neurotoxicol. Teratol*. 90:107061, 2022 for details.

Essential metals often use shared pathways for absorption and transport due to their similar chemical and structural properties. Mn and Iron (Fe) can be transported by the same carrier, divalent metal transporter-1 (Chen et al., 2015; Fitsanakis et al., 2010; Tallkvist et al., 2000), and often if one is in excess it can impact the absorption, physiological function, and the toxic effects of the other. For this reason, we chose to measure the concentrations of both Mn and Fe in maternal and offspring tissues.

Table 3.2 shows the Mn and Fe concentrations in whole blood and whole brain of the dams following cessation of exposure (PND23). Concentrations of Mn in both the blood and brain were increased in a dose-dependent manner, and Mn concentrations were higher in brain than blood in all treatment groups. Analysis also showed that stress modified the blood Mn concentrations, as perinatal-stressed dams had higher blood Mn concentrations than no-stress animals, independent of Mn dose. This stress effect was not seen in the brain Mn levels.

Table 3.2. Tissue concentrations (µg/g of tissue) of Mn and Fe in dams following cessation of exposure.

		0 mg/ml Mn		2 mg/ml Mn		4mg/ml Mn	
		NS (N=16)	**PS** (N=18)	**NS** (N=19)	**PS** (N=18)	**NS** (N=16)	**PS** (N=18)
Mn	Blood	0.009 ± 0.001	0.017 ± 0.003[c]	0.047 ± 0.004[a]	0.062 ± 0.005[ac]	0.185 ± 0.039[ab]	0.212 ± 0.057[abc]
	Brain	0.479 ± 0.026	0.445 ± 0.007	0.696 ± 0.016[a]	0.710 ± 0.015[a]	0.999 ± 0.042[ab]	0.970 ± 0.035[ab]
Fe	Blood	418.40 ± 18.80	437.15 ± 17.48	410.39 ± 21.31	398.24 ± 18.46	403.50 ± 29.85	371.56 ± 18.22
	Brain	22.56 ± 1.34[d]	20.59 ± 0.58[d]	20.47 ± 0.47	20.01 ± 0.47	19.66 ± 0.75	18.99 ± 0.43

Superscripts: Mn: a denotes P < 0.05 compared to 0 mg/mL Mn group, b denotes P < 0.05 compared to 2 mg/mL Mn group, c denotes P < 0.05 compared to NS group; Fe: d denotes p < 0.05 compared to 4 mg/mL Mn group. See Beasley et al., Neurotoxicol. Teratol. 90:107061, 2022 for details.

Mn and Fe concentrations in whole blood and whole brain in the offspring at PND2 and PND22 are presented in Table 3.3. As seen in the dams, Mn concentrations in both the blood and brain of the offspring at PND2 and PND22 were increased in a dose-dependent manner such that the two Mn-exposed groups had higher concentrations of Mn compared to the 0 mg/mL Mn group. No effect of perinatal stress on Mn or Fe concentrations was observed at either timepoint in the blood or the brain.

Table 3.3. Concentrations (µg/g tissue) of Mn and Fe in the whole blood and whole brain in offspring.

Mn		0 mg/ml Mn		2 mg/ml Mn		4mg/ml Mn	
		NS	PS	NS	PS	NS	PS
PND2	Blood	0.092 ± 0.034	0.051 ± 0.011	0.390 ± 0.044[a]	0.450 ± 0.056[a]	0.631 ± 0.056[ab]	0.664 ± 0.061[ab]
	Brain	0.323 ± 0.017	0.316 ± 0.012	0.492 ± 0.031[a]	0.492 ± 0.031[a]	0.628 ± 0.045[ab]	0.691 ± 0.039[ab]
PND22	Blood	0.015 ± 0.002	0.031 ± 0.008	0.065 ± 0.004[a]	0.087 ± 0.017[a]	0.168 ± 0.014[ab]	0.168 ± 0.014[ab]
	Brain	0.607 ± 0.019	0.594 ± 0.017	1.066 ± 0.040[a]	0.929 ± 0.039[a]	1.418 ± 0.120[ab]	1.475 ± 0.130[ab]

Superscripts: [a] denotes p < 0.05 compared to 0 mg/mL Mn group, [b] denotes P < 0.05 compared to 2 mg/mL Mn group. Tissues within a litter were combined at PND2. Tissue concentrations for male and female offspring are combined within litter at PND22.

Fe		0 mg/ml Mn		2 mg/ml Mn		4mg/ml Mn	
		NS	PS	NS	PS	NS	PS
PND2	Blood	312.90 ± 9.92[d]	315.70 ± 12.67[d]	315.78 ± 12.13[e]	312.94 ± 12.48[e]	278.68 ± 11.83	294.04 ± 12.73
	Brain	14.74 ± 0.41[df]	15.19 ± 0.48[df]	13.35 ± 0.34[e]	12.56 ± 0.34[e]	11.44 ± 0.38	12.07 ± 0.67
PND22	Blood	188.82 ± 6.88	208.14 ± 6.11	191.38 ± 6.20	193.03 ± 10.49	186.07 ± 21.04	179.48 ± 16.25
	Brain	10.68 ± 0.41[d]	10.27 ± 0.32[d]	9.42 ± 0.21	9.40 ± 0.25	8.70 ± 0.28	8.72 ± 0.38

Superscripts: [d] denotes p < 0.05 compared to 4 mg/mL Mn group, [e] denotes p < 0.05 compared to 4 mg/mL Mn group, [f] denotes p < 0.05 compared to 2 mg/mL Mn group. Tissues within a litter were combined at PND2. Tissue concentrations for male and female offspring are combined within litter at PND22. See Beasley et al., Neurotoxicol. Teratol. 90:107061, 2022 for details.

To evaluate the neurological development of the rat offspring, a battery of behavioral and cognitive tests was chosen, consisting of novel object recognition test, Morris water maze, differential reinforcement of low rates procedure, choice reaction time, ultrasonic vocalization, locomotor activity, social approach, acoustic startle response and prepulse inhibition, and sweetness preference for a chocolate flavored milk solution. Findings for individual tests are described in detail.

3.5 Evaluations of neurobehaviors in offspring

Novel object recognition test measures the rat's natural preference for novel objects. A rat is placed in an acrylic box that has two identical objects (about 30.5 cm high). The time the rat spends investigating each object and the number of visits to each object is recorded. After a period of 1 h, one object is replaced with a new "novel" object, and the time the rat spends investigating it is again recorded. Short-term memory is indicated by the rat's preference to visit and/or spend more time with the novel object. These measures are converted to a preference index which must differ from the no-preference index (0.5) to indicate memory. This test was done in early adolescents.

As seen in Figure 3.5, Mn (4 mg/mL) alone increased novel object preference time in females (A) and reduced novel object preference visits in males (B) compared to the 0 mg Mn/mL group. Similarly, perinatal stress alone (PS-0 mg/mL dose) tended to increase novel object preference time in females, while reducing preference visits in males. The effects observed in females indicate Mn and stress had faciliatory effects on short term memory at a delay period of 1 h, while in males, these two factors impaired short term memory. However, perinatal stress combined with Mn attenuated the effects of Mn in both sexes.

Figure 3.5. Effects of maternal Mn treatment on the offspring's preference for a novel object was changed by exposure to perinatal stress.

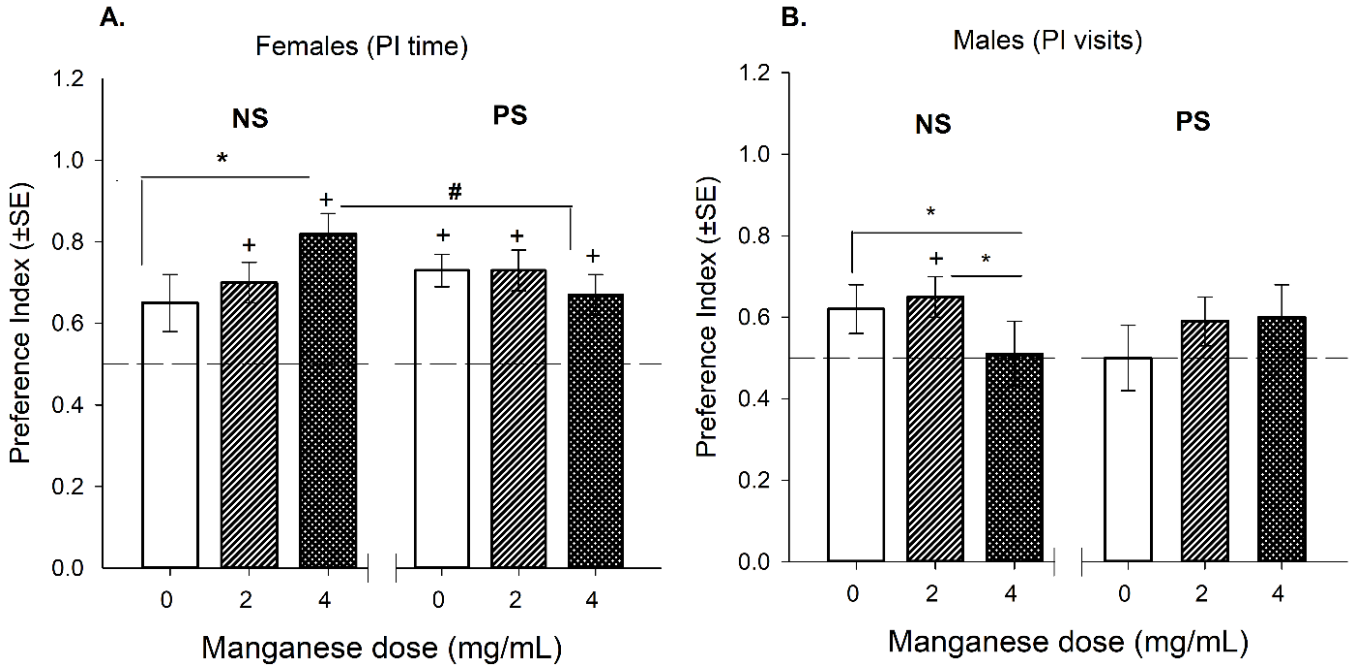

Figure 3.5. Novel object preference based on time in females (A) and visits in males (B) as a function of Mn dose group, in non-stressed (NS) or perinatally-stressed (PS) rats. (*) indicates difference between Mn doses, (#) indicates difference between stress groups, and (+) indicates preference index (PI) differs from 0.5 in t-test comparisons. Means ≤0.5 (dotted line) indicate no preference for either object. Data are presented as LSmeans ± SE. See Oshiro et al., *Neurotoxicol. Teratol.*, 91:107077, 2022 for details.

Morris water maze (MWM) is a test of learning and spatial memory. Place learning is measured by using a submerged platform and having the rat learn its location using cues around the room. Two trials per day (with a 5-min intertrial interval) are conducted over 9 days. Reference memory is conducted by removing the platform and measuring the rat's propensity to search in the area where it was located. Reversal learning is measured by moving the platform and retraining the rat to a new position, using two trials a day for 3 days.

Spatial learning and memory as assessed by the MWM test were not altered by Mn and/or stress. All animals learned the spatial task as evidenced by shortened pathlengths and latencies in females and males across the 9 test days (data not shown). Follow-up challenges after learning (reference and reversal challenges) also showed no differences between treatment groups. The lack of effects observed on the MWM tests provides strong support for intact allocentric learning processes in offspring after Mn and stress exposure. As previously observed, males learned this task better than females (Jonasson, 2005; Vorhees et al., 2008), showing that expected biological differences were detected. Mn did increase thigmotaxis (propensity to stay near the tank wall during trials) in both sexes, which can be an indicator of increased anxiety, but this did not alter learning on this task.

Differential reinforcement of low rate (DRL) task assesses spontaneous activity, timing perception, and impulsivity. The rat is trained to withhold responding for a predetermined amount of time to receive food reinforcers, and any responses before that time, resets the clock for an additional fixed time. Daily sessions consist of 100 correct responses or 60 min, whichever occurs first. Testing occurs in weight-maintained adults.

As part of training for the DRL, rats must first learn to press a lever using an autoshaping-operant method previously described (Davenport, 1974; Samsam et al., 2005), in which the retraction of a lever is paired with the delivery of a food pellet. Over the course of 5 days, rats learn to press the lever on their own as indicated by an increase in lever press responses across blocks and days of testing. Mn (2 mg/mL) had a faciliatory effect on lever press responses in both sexes on day 4 of training during blocks 2 to 5 of autoshaping (Figure 3.6 A). In contrast, perinatal stress impaired lever press responses on days 3 to 5 of autoshaping in males and females combined (Figure 3.6 B). Although an interaction occurred between Mn and stress, additional analyses did not reveal differences between the treatment groups. Taken altogether, stress and Mn worked counteractively on learning of the lever press response.

Figure 3.6. Effects of maternal exposure to 2 mg/mL Mn and stress altered behavioral responses by adult offspring during the acquisition of the differential reinforcement of low rate (DRL) task.

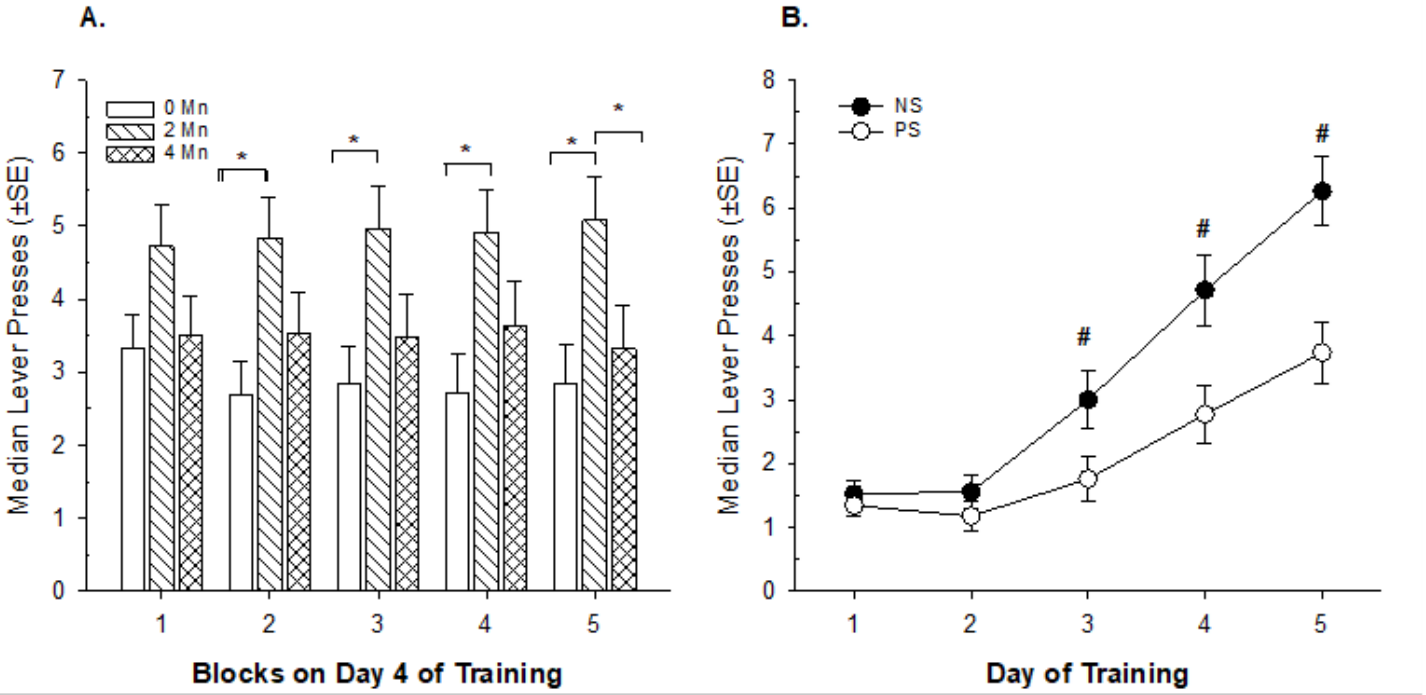

Figure 3.6 A-B. Lever presses during autoshaping procedures (with sexes combined) plotted as a function of Mn and stress dose group on day 4 (A) and stress group (B): non-stressed (NS) and perinatal-stress (PS) across days of learning. (*) indicates differences between Mn groups, (#) indicates differences between stress groups on days indicated (all p-values<0.05). Data are presented as means ± SE. See Oshiro et al., *Neurotoxicol. Teratol.*, 91:107077, 2022 for details.77.

During the DRL test with a 10-s delay, except for efficiency on day 1 of learning where stressed females performed better than non-stressed females, no treatment-related effects were observed on timing errors, bursting, or efficiency during steady-state performance of the task. These results indicated no evidence of impulsive responses during the 10-s delay period or timing deficiencies in animals treated with Mn and/or stress.

Choice reaction time task (CRT) assesses attention, impulsivity, and reaction time. The choice reaction time task is initiated with a nose poke into a central port for a variable hold period. A tone is used as a stimulus to alert the end of the hold period, and the rat must then move to an adjacent alcove. On cued trials, the tone is preceded by a light in the correct response alcove, and on uncued trials the tone is presented at the same time as the light in the correct response alcove. On cued trials, the light in the correct alcove is extinguished at the end of the hold period, and, on uncued trials, the light in the correct alcove is extinguished when the rat removes its nose from the central port. Five daily sessions are given for each trial type and end after 100 correct responses or 60 min, whichever occurs first. Testing occurs in weight-maintained adults.

Mn and stress impacted CRT performance measures similarly, as both factors reduced accuracy (Figure 3.7 A), increased anticipatory responding (Figure 3.7 B), and slowed reaction time measures: decision time (Figure 3.7 C) and movement time (Figure 3.7D). These effects were sex and task specific. Interactions between Mn and stress occurred predominantly by altering the 0 mg /mL Mn dose effects. For each measure during cued CRT, a change occurred in the 0 mg /mL Mn dose combined with stress; this included reduced accuracy (Figure 3.7 A), increased anticipatory responding, and slowed reaction times. Two measures yielded sex-specific changes after treatment with Mn and stress (accuracy and movement time), whereas anticipatory responding and decision time were similarly altered in both sexes. Perinatal stress attenuated the Mn effects on these CRT measures.

Figure 3.7. Both Mn and perinatal stress treatments altered performance of adult offspring in the choice reaction time task.

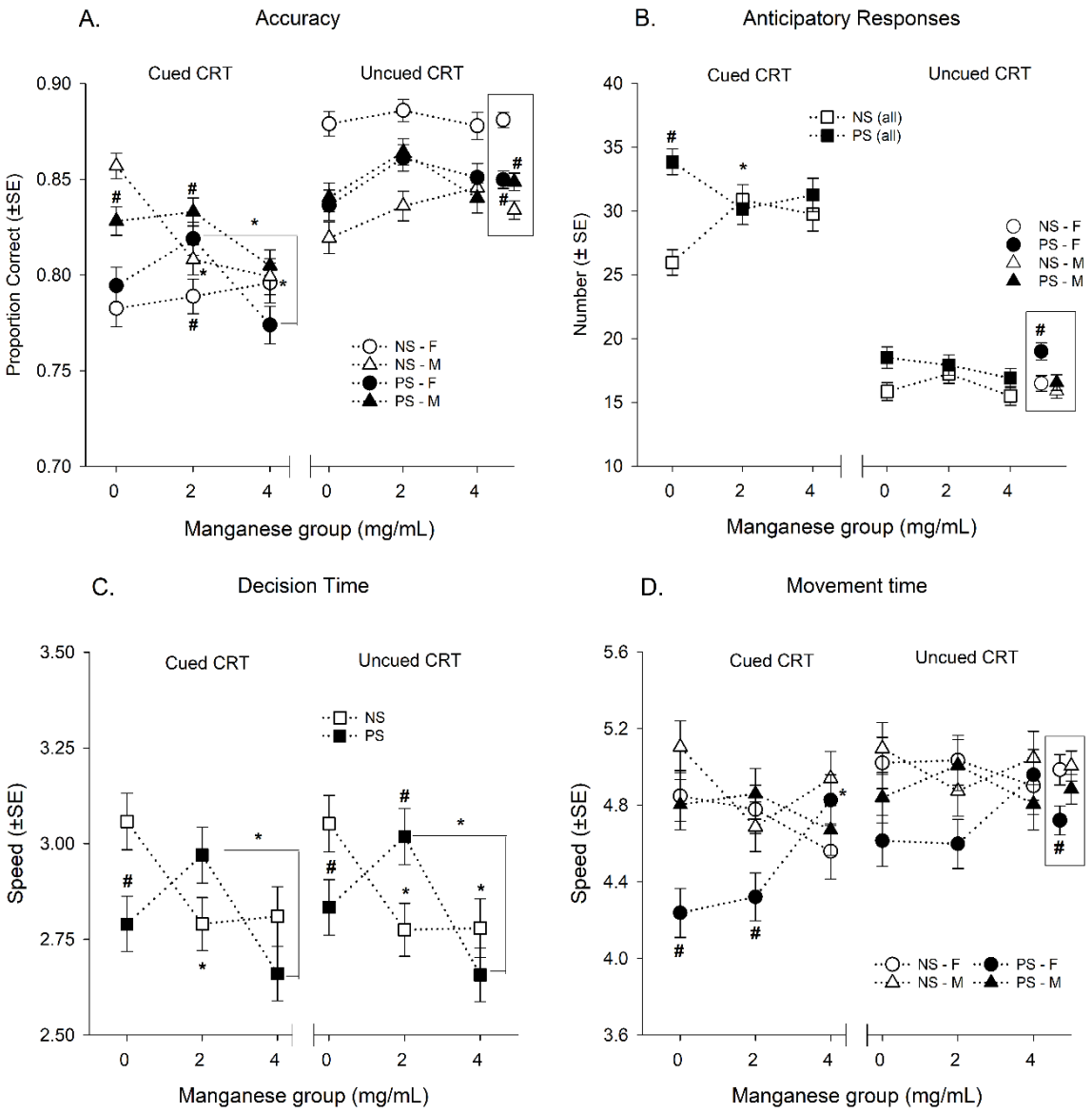

Figure 3.7 A-D. Performance measures during cued and uncued CRT as a function of treatment group. Dotted lines indicate between-group effects of Mn within the same stress group: non-stressed (NS) or perinatally-stressed (PS). Cued accuracy (A, left) was impaired by Mn and stress in males but not females, but when combined, stress improved accuracy in both males and females in the 2 but not the 4 Mn dose group. During uncued CRT (A, right), accuracy in females was impaired by stress, while males were improved by stress (collapsed across Mn dose) (A, inset). Anticipatory responses during cued CRT (B, left) were increased by Mn and stress with sexes combined. During uncued CRT (B, right), PS females had increased anticipatory responding compared to NS females (B inset). Decision times during cued CRT (C, left) and uncued CRT (C, right) were slowed by Mn (compared to 0 mg/mL) and by stress in the 0 mg/mL group. When combined, stress improved decision speed in the 2 but not the 4 mg Mn/mL group. Movement times during the cued CRT (D, left) were slowed by stress in the females only, but when combined with the 4 mg Mn/mL dose the effect of stress was attenuated. During uncued CRT (D, right, inset) stress slowed movement time in females but did not interact with dose. All values are LSMeans. (*) indicates differences between Mn doses indicated; (#) indicates differences between stress groups for a given Mn dose (all p-values<0.05). See Oshiro et al., *Neurotoxicol. Teratol*., 91:107077, 2022 for details.

Social approach task examines the animal's social behaviors. A rat is placed in an acrylic box and separated from a stimulus rat by a perforated divider. The number of visits and time spent interacting with the stimulus rat is recorded as a measure of sociability. This is conducted in adolescents.

As shown in Figure 3.8, male control rats had less preference for a novel stimulus rat when compared with the 4 mg/mL Mn-exposed group. This effect in males suggests the Mn-exposed rats preferred to spend time with the stimulus rat versus the empty side of the box. There was also a stress by sex interaction for the preference index of time spent with the stimulus rat where the non-stressed females spent more time than the non-stressed males.

Figure 3.8. Maternal treatment with Mn increased the preference for a stimulus in male but not female offspring.

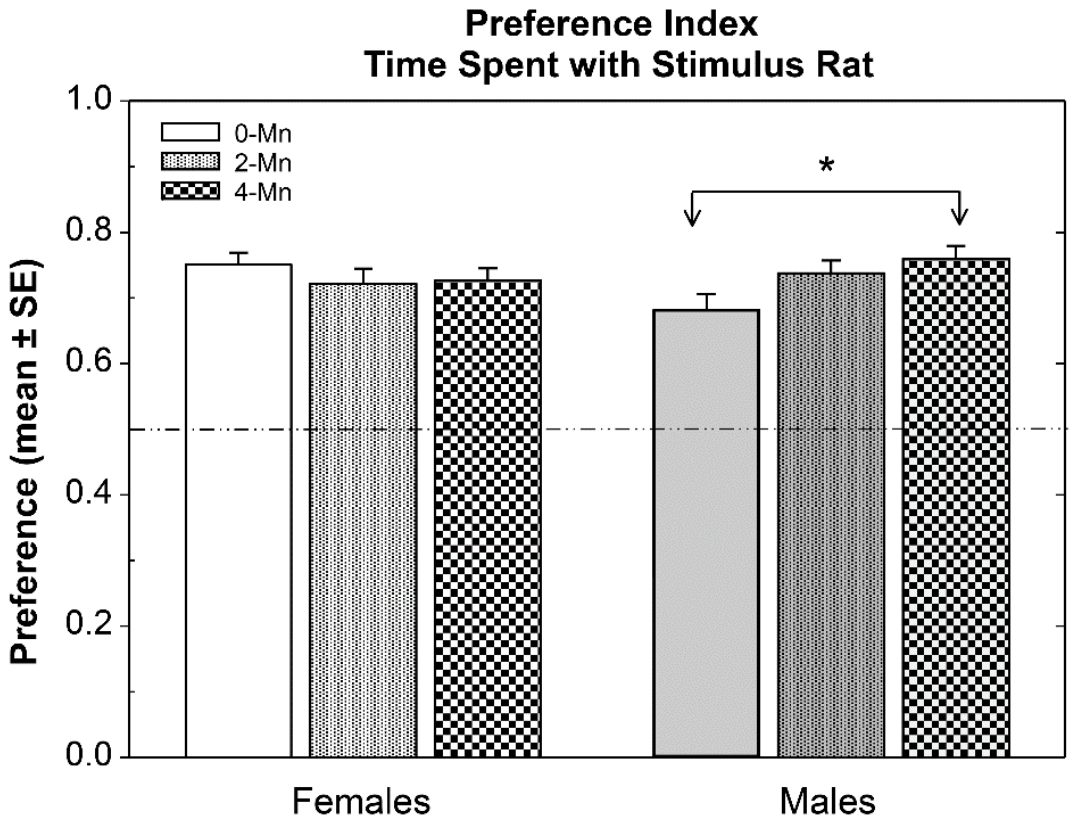

Figure 3.8. Preference based on proportion of time spent visiting the stimulus rat during the social approach test collapsed across stress. The 4 mg/mL Mn males spent more time with the stimulus rat compared with the control males (denoted by *). The segmented line represents equal preference. See McDaniel et al., *Neurotoxicol. Teratol.* 91:107088, 2022 for details.

Acoustic startle response is the amplitude of the muscular response (flinch) to a sudden, loud (115-dB) burst of sound. Habituation to the startle noise is measured across 40 trials. The startle reflex can be modified with white noise delivered shortly before (prepulse) the burst, which decreases the magnitude of the startle. This change in startle amplitude is known as prepulse inhibition (PPI) and is a measure of sensory-motor gating. Following the 40 habituation trials, 60 trials are given to assess PPI. Thus, a total of 100 trials are given during each test session. This test is conducted in late adolescent rats.

There were no changes in startle amplitude, habituation to the startle stimulus, or the ability of the prepulse to decrease the subsequent startle response associated with the Mn and/or stress treatments (data not shown). This suggests the treatments produced no major changes in startle reactivity and habituation or sensory-motor gating.

Locomotor activity is measured in automated chambers shaped liked a figure 8. The locomotor activity and rearing are recorded over the test session. Testing occurs at PND17 (horizontal activity only) and again in adolescents (PND29) and adults (PND79).

There was no effect of Mn or stress on habituation or on total activity counts at any age tested. However, there was an effect of Mn on horizontal counts at PND29 where the 2 mg/mL group had greater counts than the 0 mg/mL group (Figure 3.9).

Figure 3.9. Maternal treatment with 2 mg/mL Mn increased motor activity in young offspring.

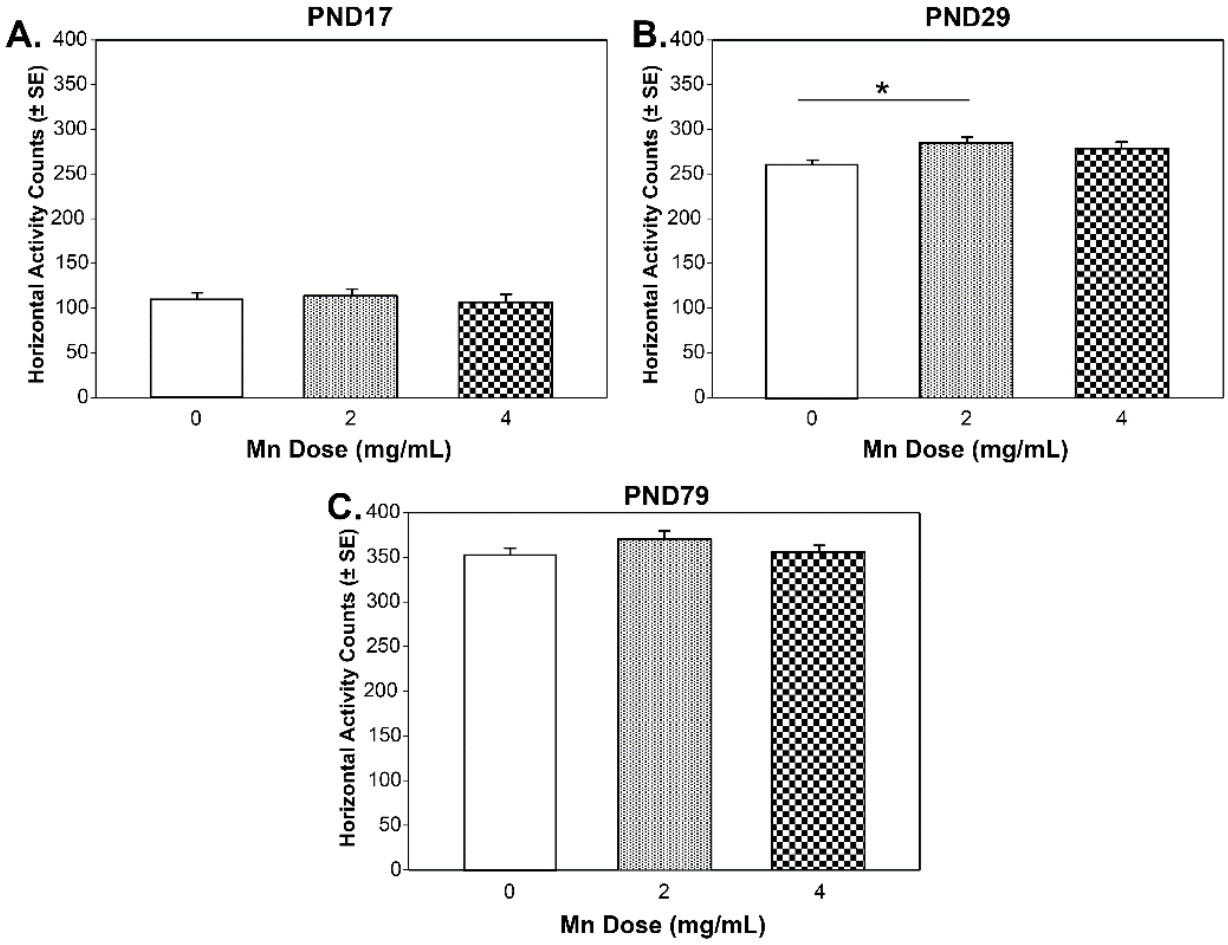

Figure 3.9. Horizontal activity counts at the three ages tested, collapsed across sex. On PND29 the 2mg/mL Mn group had greater horizontal activity compared with the 0 mg/mL Mn group (denoted by *). Data are presented as means ± SE. See McDaniel et al., *Neurotoxicol. Teratol.* 91:107088, 2022 for details.

Ultrasonic vocalizations (USVs): Calls are emitted by rats which are above the human threshold of hearing (>20 kHz). These calls have been classified into three distinct classes based on their average frequency: (1) 40 kHz: emitted by pups, usually during stressful situations (separation from mother and litter and during a drop in ambient temperature); (2) 22 kHz: emitted by adults, usually during a threat or elevated anxiety; and (3) 50 kHz: emitted by juveniles and adults during play and appetitive behaviors. These calls provide information on the affective state of the animals, as well as their communication with dam and conspecifics at various life stages. With the advancement of specialized hardware and software in recent years, the pup calls have been further categorized based on call characteristics (e.g., frequency) (Boulanger-Bertolus et al., 2017). We examined calls emitted by pups on PND13 during 3 min of maternal separation. We classified these calls into three categories: (1) 40 kHz (calls <45 kHz, possible precursors to the 22-kHz call observed in adults), (2) 60 kHz (calls >45 kHz, possible precursors to the 50-kHz calls observed in juveniles and adults), and (3) frequency-modulated calls (FMC; calls with more than one element that differed by more than 5 kHz between adjacent elements).

Figure 3.10. Female offspring perinatally exposed to 4 mg/mL Mn perinatally had increased modulation of ultrasonic vocalizations.

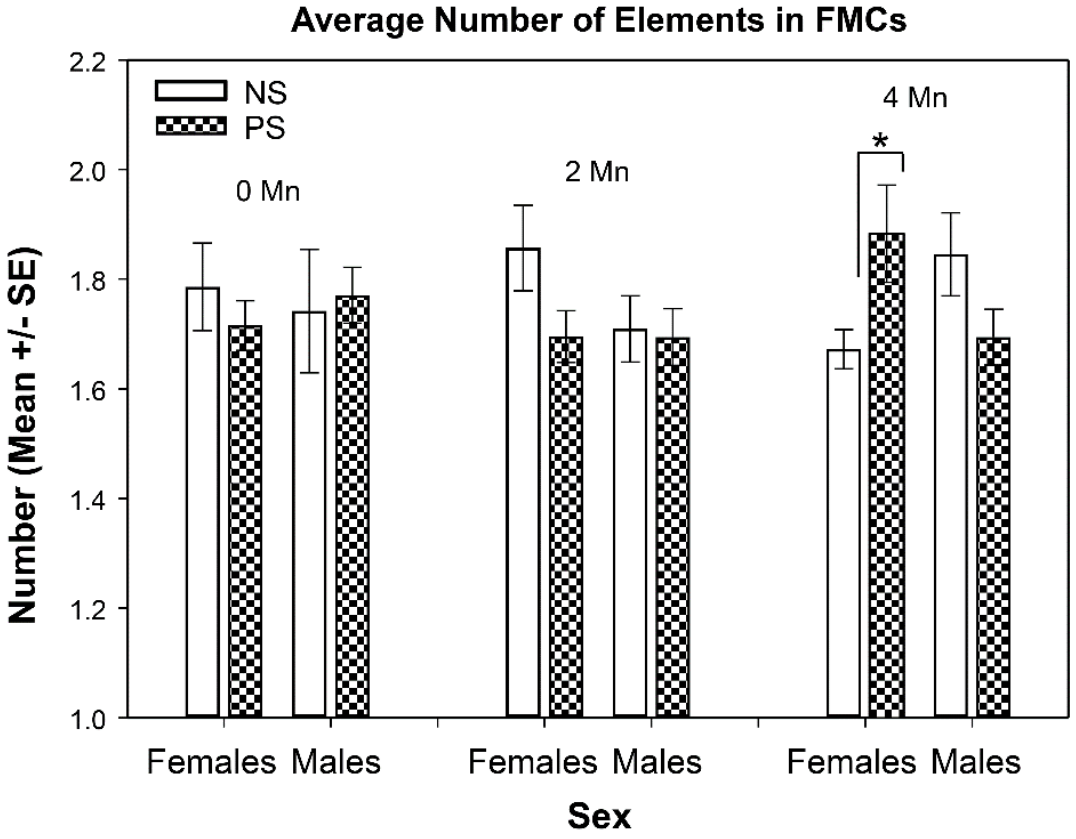

Figure 3.10. The number of elements in frequency-modulated calls (FMCs) was increased in female offspring perinatally treated with 4 mg/mL Mn. Data are presented as means ± SE. See McDaniel et al., *Neurotoxicol. Teratol.* 91:107088, 2022 for details.

Traditional measures of ultrasonic vocalizations for calls in the 40- or 60-kHz ranges were not altered by the Mn and/or stress treatments. However, a newly recognized call type that modulates between 40 and 60 kHz was altered by Mn treatment (Figure 3.10). Female pups had a greater number of modulations per call after exposure to 4 mg/mL Mn. This result suggests a different state of emotionality in these young female animals.

Anhedonia assesses the loss of interest in a normally rewarding stimulus (generally accepted as belonging to the spectrum of depression). This emotionality behavior was evaluated by measuring consumption of a chocolate flavored milk solution, which typically is preferred by rats. The rats have a choice of two bottles (water and chocolate milk) for 2 h closest to the dark cycle, and sweetness preference is assessed by relative consumptions. This is conducted in late adolescents.

There was no effect of Mn or stress on the sweetness preference for the chocolate flavored milk solution when body weight was considered in either sex. All groups had a preference of 90% or greater (data not shown).

3.6 Discussion

In this study, we examined potential interactions in effects of perinatal exposure to Mn and concurrent nonchemical stressors on offspring. Serum CORT levels indicated that the experimental manipulations were able to elevate stress levels in the dams, regardless of Mn level. However, the maternal CORT levels did not differ between treatment groups after birth (PND9). Tissue Mn concentrations in the brain and blood were elevated in the dams at PND23, and the addition of stress further increased Mn levels in the blood. The Mn concentrations in both the blood and brain of the offspring were also increased in a dose-dependent manner at PND2 and PND22, but unlike the dams, there was no effect of perinatal stress on Mn concentrations at either timepoint in the brain or blood. Mn (4 mg/mL) reduced maternal weight gain during gestation and offspring weight gain up to PND34. However, stress did not alter these effects. Manganese affected several measures of behavior and cognitive function, indicating a long-term impact of the Mn exposure. Many behavioral endpoints at different ages that were task specific (locomotor activity, sociability in males, velocity, distance traveled, and thigmotaxis) were affected by Mn, indicating altered motor output and affective states in offspring, but there was no effect of stress alone (except for the stress by sex effect which was only in the no-stressed groups) or in combination with Mn on these endpoints. In contrast, both Mn and stress impacted cognitive functions in offspring (short-term memory, lever-press learning, attention, impulsivity, and reaction time measures). More importantly, the combination of these two factors exerted a different profile of effects compared with either factor alone. For most of these combined effects, the two factors did not show increased effects but either attenuated or reversed the effects of the other treatment, even when the effect of each factor alone were in the same direction. The effects of stress on control performances of the choice reaction time assessments and its attenuation of effects of Mn in the novel object memory test are of concern because these interactions reduce the ability to detect changes caused by Mn and, potentially, other suspected developmental neurotoxicants in vulnerable populations.

3.7 Conclusions

Perinatal stress did not exacerbate the effects of Mn in this study. However, especially for higher order cognitive functions, perinatal stress changed the response to Mn. This interaction resulted in no discernable effect of Mn on some of the measures. The results we observed, as well as those described in the literature, indicate that more consideration should be given to multidimensional models of developmental toxicant exposure, in this case Mn co-occurring with environmental risk factors such as stress. Given the significance of the mother-infant interactions, understanding how chemical and nonchemical stressors lead to alterations in fetal brain chemistry and behavioral development should be of utmost concern.

3.8 References

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Section 4. Topical literature reviews

Three literature reviews were conducted to support the tenets of this research. This study hypothesized that maternal factors may interact with exposures to chemicals during pregnancy and early life in producing long-lasting deleterious health effects in offspring. The first review summarized the current state of the science in epigenetics, which represents a key mechanism by which the intrauterine environment could influence the risk of adult disease in offspring. This field is advancing rapidly, and this review was done to bring our team up to speed and to understand how epigenomic changes might be tied to chemical or nonchemical exposures during development. The second review examines the effects of a known teratogen, tobacco smoke, on the epigenome of children exposed to maternal smoking during development and the pattern of health effects for which these children are at higher risk. This literature provides the best example of the DOHaD hypothesis in humans and is a proof of concept for both epigenetic impacts of chemical exposures in utero and related later life effects. The third review was of our current understanding of the susceptibility of the in utero and early life periods to the induction of cancer caused by chemical exposure. This review was undertaken in response to interest expressed by the EPA Science and Technology Policy Council and because the topic was relevant to our research. These three reviews will be discussed individually in the following subsections.

4.1 Epigenetics and the Developmental Origins of Health and Disease

The primary DNA sequence is the foundation for understanding the genetic program. Superimposed on the DNA sequence is a layer of heritable "epigenetic" information. Epigenetics has been defined as "mitotically and/or meiotically heritable changes in gene function that cannot be explained by changes in DNA sequence" (Russo et al., 1996). Epigenetics is the study of chromatin structures and DNA modifications that determine which genes are expressed and which are not. Epigenetic mechanisms at the level of chromatin include chemical modifications falling into two main categories: (1) DNA methylation (Smith and Meissner, 2013; Messerschmidt et al., 2014), and (2) posttranslational modifications (e.g., acetylation, methylation, phosphorylation) of the histone proteins that package the genome (Bernstein et al., 2007). By regulating DNA accessibility and chromatin structure, these chemical changes influence how and when the genome is transcribed across diverse developmental stages, tissue types and disease states (Bird, 2002; Goll and Bestor, 2005; Margueron et al., 2005; Adalsteinsson and Ferguson-Smith, 2014). In addition, noncoding RNAs have been considered as epigenetic in nature and can act at the level of transcription, translation, or posttranslationally (Cech and Steitz, 2014).

Rogers et al. (2018) reviewed the mechanisms of epigenetics, patterns of epigenetic changes during gametogenesis, and early development and elaborated the role of epigenetics in the developmental origins of health and disease. They described the current understanding of the enzymes (e.g., DNA methyltransferases, histone deacetylases) that operate to modify DNA and histones to alter the epigenome. The pluripotent cells of the cleavage-stage conceptus progressively differentiate along specific lineages to give rise to the tissues of the embryo and fetus. Although regulation of differential gene expression by transcription factors is a key feature of development, it is now understood that gene expression patterns during development depend on epigenetic modifications (Li, 2002; Morgan et al., 2005), including those described above: methylation of DNA (Bird, 2002; Li, 2002), posttranslational modification of histone protein tails (Li et al., 2007; Turner, 2007), and noncoding RNAs (Ringrose and

Paro, 2004). These epigenetic “marks” may be transient, such as the histone modifications that, during cleavage, repress genes needed for later development, or long-lived, such as the DNA methylation and other chromatin modifications that result in X-chromosome inactivation or the silencing of imprinted genes.

Epigenetic marks may be erased and reestablished at specific stages of the life cycle. There are two periods during which large-scale demethylations of the genome are known to occur (Sasaki and Matsui, 2008). One is during migration and proliferation of the primordial germ cells (PGCs), which takes place between embryonic days 10.5 and 12.5 in the mouse. During this period, the imprinted genes are demethylated (Hajkova et al., 2002; Li, 2002). Methylation is reestablished later, in a parental gender-specific manner, during gametogenesis by DNA methyltransferases. Genomic demethylation is almost complete in PGCs (Lane et al., 2003).

The other period of widespread epigenetic reprogramming occurs early after fertilization. The sperm genome is among the most highly methylated of any cell type in the mouse, yet after fertilization, many paternal genes become demethylated. This active demethylation of the paternal genome before the onset of DNA replication is followed by passive demethylation of both parental genomes by dilution once rapid DNA synthesis and cleavage begins. Total genomic methylation in the early embryo decreases, reaching a nadir at the blastocyst stage.

There is now compelling epidemiological and laboratory experimental evidence that the in utero environment in which a conceptus develops, as well as the early postnatal environment, affects the lifelong health and disease susceptibility of the offspring (Gluckman et al., 2007; Godfrey et al., 2007; McMillen and Robinson, 2005; Nathanielsz et al., 2007; Ozanne and Constancia, 2007; Fall, 2013). It is likely that the patterns and extent of epigenetic marks on the genome may be specified or altered, in part, by the developmental environment. Because these epigenetic marks can last a lifetime, it is plausible that epigenetic programming during development results in permanent changes in the physiology and, therefore, adult disease risks of the offspring. The potential role of developmental epigenetic programming in later risk of disease is an area of intense investigation.

Epigenetic changes during development have been studied most frequently in the context of maternal nutritional deficiencies or undernutrition. Offspring of mothers undernourished during pregnancy are born small and have a higher risk of later life obesity (Ravelli et al., 1999), diabetes (Ravelli et al., 2000), kidney disease (Painter et al., 2005) and coronary heart disease (Roseboom et al., 2001). Although the evidence for maternal diet affecting the epigenome of her offspring is strong, it has more recently come to light that exposure to chemical and nonchemical stressors (e.g., psychosocial stress) during pregnancy can have long-term effects on offspring, much like those seen in offspring of undernourished mothers. Rogers et al. (2018) summarized the evidence supporting epigenetic programming by chemical and nonchemical stressors, including vinclozolin, bisphenol A, metals, therapeutic drugs, maternal behaviors, and assisted reproduction technologies.

4.2 Epigenetic consequences of maternal smoking during pregnancy, and latent health effects in offspring

The best example of an exposure during pregnancy in humans resulting in epigenetic changes and elevated risk of metabolic disease in offspring is maternal smoking. Rogers (2019) reviewed the involvement of epigenetics in the adverse health outcomes from smoking during pregnancy. Maternal smoking causes lower birth weight, birth defects, and other adverse pregnancy outcomes. The latent and persistent metabolic effects in offspring of smoking mothers resemble those observed in studies of maternal undernutrition during pregnancy. Offspring of smoking mothers, like those of undernourished mothers, are born smaller than those of nonsmoking mothers and have higher risk of obesity or overweight by adolescence. Altered patterns of DNA methylation have been documented consistently in smoking mothers' offspring, and the extent of these epigenetic alterations are extensive and postnatally persistent. A causal link between altered DNA methylation and the phenotypic changes observed in offspring remains to be firmly established, yet the association is strong, particularly for lower birthweight. The adverse effects of exposure to tobacco smoke during pregnancy now clearly include permanent metabolic derangements in offspring that can adversely affect life-long health.

4.3 Prenatal chemical exposure and the risk of childhood cancer

The lability of the epigenome prenatally may increase the sensitivity of the developing conceptus to DNA damage or adverse epigenetic changes. There is evidence that the prenatal period is susceptible to the induction of cancers that manifest in childhood or later. However, which types of cancers are more likely with prenatal exposures or which carcinogens are more potent during the in utero period are poorly understood. Vulimiri and Rogers (2018) examined whether children are more vulnerable than adults for cancer induced by in utero exposure to xenobiotics. They evaluated the potential mechanisms of prenatal cancer induction, including the emerging concept of epigenetic programming during early life. They described several case studies to highlight the diverse types of prenatal exposures that increase cancer risk later in life, including radiation, diethylstilbestrol, tobacco smoke, pesticides, and arsenic, and cancer types, such as breast cancer and leukemia. They concluded that there is ample evidence to support the idea that prenatal exposure to carcinogens is sufficient to induce cancer later in life in offspring from both humans and experimental animals. The prenatal period may be more, similarly, or less sensitive to the induction of cancer from chemical exposures than the adult, depending on the nature of the carcinogen. Environmental chemicals act through different mechanisms in the developing conceptus, dependent on both chemical class and the timing of in utero exposure. Depending on whether carcinogens interact directly or indirectly with the genetic material, they are called genotoxic or nongenotoxic carcinogens, respectively.

Genotoxic chemicals interact with DNA and can alter the DNA bases, whereas nongenotoxic chemicals do not interact with DNA directly, but are believed to act by altering the rate of cell proliferation or by mechanisms that enhance the risk of genetic errors (Eastmond, 2012). Among the genotoxic chemicals, some are highly reactive, hence the parent compound can bind directly to the genetic material (direct acting), whereas others require metabolic activation by xenobiotic metabolizing enzymes, and one or more of the metabolites formed from the parent compound bind to DNA (indirect acting). It has been reported that many, but not all, carcinogens cross the placental barrier (Autrup, 1993). Further, in comparison to indirect-acting genotoxic chemicals, certain direct-acting genotoxic chemicals, such as alkylating agents, appear to be more potent in cancer induction during early embryogenesis. For the

indirect-acting chemicals to be transplacentally carcinogenic, the ultimate carcinogenic metabolite formed in the maternal tissue must be stable enough to reach the fetus, or the carcinogenic metabolite may be formed in the fetal tissues (Rice, 1979).

Some genotoxic environmental chemicals may form DNA adducts in fetal cells, whereby these cells are initiated, but remain dormant, until they are exposed to a cancer promoter during subsequent life stages. Such interaction may cause cellular proliferation and fix a mutation following DNA replication (Poirier, 2016). Thus, prenatal exposure to environmental chemicals may pose a risk of developing cancer in later life, in part, because of the longer period available for initiated cells to undergo tumor promotion. Further, because the growing embryo/fetus displays rapid cell proliferation in utero, susceptibility to carcinogens may be increased because of enhanced fixation of mutations with little time available for repair of carcinogen-induced DNA damage leading to clonal expansion of mutant cells, giving a larger and possibly diverse population of mutations (Anderson et al., 2000). Also, some chemicals may act as cancer initiators, whereas others may act as tumor promoters; it is likely some chemicals may act as both initiators and promoters. Thus, in rapidly dividing cells, transplacental carcinogens might induce DNA damage, chromosomal instability because of alterations in the genetic material, or mutations, eventually advancing the carcinogenic process.

Unlike genotoxic agents, nongenotoxic chemicals do not directly interact with genetic material. For example, endocrine-disrupting chemicals can act through mechanisms inducing hormone-sensitive cancers in women (Scsukova et al., 2016) and cancer of the prostate gland in men (Gore et al., 2015). Toward this end, a literature search was conducted in PubMed using a list of key words, such as “cancer or neoplasms” and “in utero or prenatal or transplacental.” Reports of regulatory and nonregulatory agencies were searched for information on prenatal exposures to putative carcinogens from the agency Web sites. The purpose of this minireview was to examine the current views on in utero exposure to environmental carcinogens and how this information may be considered for health risk evaluation.

An interesting finding pointing to an epigenetic mechanism of carcinogenesis is the fact that paternal smoking is more strongly linked to offspring cancers than is maternal smoking. The contribution of the father’s sperm is only his genome, so it is likely that either mutations or epimutations underlie the increased risk of cancer in offspring of smoking fathers. The biology underlying life stage differences in susceptibility undoubtedly include both pharmacokinetic and pharmacodynamic differences. Our growing understanding of reprogramming of the epigenome during gametogenesis and early embryogenesis and elucidation of epigenetic changes occurring in carcinogenesis will be instrumental for development of predictive biomarkers for later life risk of cancer from prenatal exposures, enabling prospective identification of at-risk populations.

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Section 5. Summary and Conclusions

This EPA research addresses the sensitivity of vulnerable groups, with a primary focus on early life stages and interactions of exposures to environmental chemicals with modifiable factors, such as diet (e.g., "bad" high-fat or high-fructose maternal diets during pregnancy; "good" diets supplemented with essential fatty acids), lifestyle (active versus sedentary), maternal obesity, and maternal psychosocial stress. These interactions model the human conditions and are happening every day in communities across the country. The studies in this report modeled some of the most common modifiable factors seen in almost every community in the United States, and especially in underprivileged communities, including maternal obesity, poor diets, and psychosocial stress. Animal models of interactions between chemical and nonchemical stressors, especially during pregnancy, have rarely been attempted because of the complex study designs required and the limited number of potential interacting factors that can be included in such studies. This research comprises studies that establish new or extend existing models for this purpose, including a variable stressor model to mirror human psychosocial stress, pre-pregnancy obesity in female rats, active versus sedentary pregnant rat models using running wheels, and poor or healthful diets. Elucidating interactions between these modifiable factors and pollutant exposures provides causal linkages to support human and epidemiological studies of these interactions, with the goal of improving community decision making about best practices to improve human health. The complex nature of these studies required animal models to incorporate these distinctly human modifiable factors.

Several studies used the ubiquitous air pollutant, ozone, as the prototypic chemical exposure. Previously unknown toxicity to early embryonic development was discovered in seminal papers by Miller and colleagues (Miller et al., 2017; 2019a,b). Exposures of pregnant rats to ozone for just 2 days during the period of embryo implantation resulted in intrauterine growth retardation that was shown to result from reduced blood flow to the uterus and effects on maternal circulating cytokines that were inhibitory to implantation. This early gestational period was not known previously to be a sensitive period for growth retardation, and this finding provides an impetus for future clinical and epidemiological investigations. In addition to this early period of sensitivity, we found that adolescent and young adult rats were more sensitive to the pulmonary toxicity of ozone than were older adults (Snow et al., 2016). Further, our findings support the beneficial effects of exercise and healthy diet in mitigating effects of ozone (and potentially other environmental pollutants) that can be directly translated to human application in affected communities. Conversely, sedentary lifestyle and poor diet were deleterious for some outcomes with exposures at different life stages, substantiating the current messages advocated by public health organizations. Although it may be intuitive that exercise and healthy diet are beneficial, whereas sedentary lifestyle, poor diet, and obesity are not, there are few studies of the interaction of these modifiable factors with exposures to environmental pollutants. Our findings demonstrate in controlled animal experiments that there are clear benefits to be gained in terms of mitigating the adverse effects of environmental exposures by modifying common lifestyle factors.

Our studies on maternal psychosocial stress required extensive effort to develop an animal model. The stress axis in humans and rats is activated in a consistent manner regardless of the nature of the actual stressor (e.g., poverty, violence, or poor living conditions in disadvantaged communities). In our model, we used a variety of moderately stressful conditions to maintain a level of stress in pregnant and

lactating rats that simulates human scenarios. This is necessary because rats are quite adaptable, and response to any individual stressor will attenuate with repeated exposures. Our model used stressors, including noise, altered light cycles, crowding, decreased bedding, and others that are varied on successive days to maintain a moderate level of stress, as confirmed by consistently elevated circulating stress hormones. The model is now well-established in our laboratory for investigation of stress effects in future experiments. Our studies examined the interactions between maternal stress and exposure to a known developmental neurotoxicant, manganese (Mn), in drinking water. This route of exposure is rarely used because the concentration of Mn in drinking water is limited by unpalatability of high concentrations of the chemical. Yet, this route is most translatable to human exposures in contaminated communities. We have documented effects of maternal stress on pregnancy outcomes and neurobehavioral development of offspring, the developmental effects of manganese, as well as the interactions of these two stressors.

In summary, our findings provide information for public health officials and community leaders to improve environments and develop sound strategies for children's healthy development, and to mitigate health disparities derived from exposures to environmental pollution. Although we did not measure epigenetic changes in the laboratory studies, it is clear from our literature reviews that this is a plausible and potentially powerful mechanism underlying adverse effects from exposures to chemical and nonchemical stressors during development. In future studies, epigenetic changes induced by in utero and early postnatal environmental factors will be a focus of our work, including as key events in adverse outcome pathways for effects on growth, physiology, and metabolism of offspring.

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Appendix A

The following is a list of individual publications in peer-reviewed journals and book volumes that support this research endeavor.

Paper #1: Snow SJ, Gordon CJ, Bass VL, Schladweiler MC, Ledbetter AD, Jarema KA, Phillips PM, Johnstone AF, Kodavanti UP. Age-related differences in pulmonary effects of acute and subchronic episodic ozone exposures in Brown Norway rats. Inhalation Toxicology. 2016; 28:313-323. doi: 10.3109/08958378.2016.1170910.

Paper #2: Gordon CJ, Phillips PM, Johnstone AFM, Beasley TE, Ledbetter AD, Schladweiler MC, Snow SJ, Kodavanti UP. Effect of high-fructose and high-fat diets on pulmonary sensitivity, motor activity, and body composition of Brown Norway rats exposed to ozone. Inhalation Toxicology. 2016; 28:203-215. doi: 10.3109/08958378.2015.1134730.

Paper #3: Miller CN, Dye JA, Ledbetter AD, Schladweiler MC, Richards JH, Snow SJ, Wood CE, Henriquez AR, Thompson LC, Farraj AK, Hazari MS, Kodavanti UP. Uterine artery flow and offspring growth in Long-Evans rats following maternal exposure to ozone during implantation. Environ. Health Perspect. 2017; 125:127005-1-127005-9. doi: 10.1289/EHP2019.

Paper #4: Gordon CJ, Phillips PM, Ledbetter A, Snow SJ, Schladweiler MC, Johnstone AFM, Kodavanti UP. Active vs. sedentary lifestyle from weaning to adulthood and susceptibility to ozone in rats. Am. J. Physiol. Lung Cell. Mol. Physiol. 2017; 312:L100-L109. doi: 10.1152/ajplung.00415.2016.

Paper #5: Gordon CJ, Phillips PM, Johnstone AMF, Schmid J, Schladweiler MC, Ledbetter A, Snow SJ, Kodavanti UP. Effects of maternal high-fat diet and sedentary lifestyle on susceptibility of adult offspring to ozone exposure in rats. Inhalation Toxicol. 2017; 29:239-254. doi: 10.1080/08958378.2017.1342719.

Paper #6: Moser VC, McDaniel KL, Woolard EA, Phillips PM, Franklin JN, Gordon CJ. Impacts of maternal diet and exercise on offspring behavior and body weights. Neurotoxicol. Teratol. 2017; 63:46-50. doi: 10.1016/j.ntt.2017.07.002.

Paper #7: Snow SJ, Cheng WY, Henriquez A, Hodge M, Bass V, Nelson GM, Carswell G, Richards JE, Schladweiler MC, Ledbetter AD, Chorley B, Gowdy KM, Tong H, Kodavanti UP. Ozone-induced vascular contractility and pulmonary injury are differentially impacted by diets enriched with coconut oil, fish oil, and olive oil. Toxicol. Sci. 2018; 163:57-69. doi: 10.1093/toxsci/kfy003.

Paper #8: Vulimiri SV, Rogers JM. Developmental origins of cancer. In Waters MD, Hughes CL (Eds.), Translational Toxicology and Therapeutics: Windows of Developmental Susceptibility in Reproduction and Cancer. John Wiley & Sons Inc., First Edition. 2018; 111-145. doi: 10.1002/9781119023647.

Paper #9: Rogers JM, Lau C, Ellis-Hutchings RG. Epigenetics and the developmental origins of health and disease. Chapter 5, Charlene McQueen (ed.), Comprehensive Toxicology, 3rd Edition. ELSEVIER, AMSTERDAM, Holland, 5:118-136, 2018. https://doi.org/10.1016/B978-0-12-801238-3.99483-2.

Paper #10: Miller CN, Stewart EJ, Snow SJ, Williams WC, Richards JH, Thompson LC, Schladweiler MC, Farraj AK, Kodavanti UP, Dye JA. Ozone Exposure During Implantation Increases Serum Bioactivity in HTR-8/SVneo Trophoblasts. Toxicol. Sci. 2019; 168:535-550. doi: 10.1093/toxsci/kfz003.

Paper #11: Miller CN, Kodavanti UP, Stewart EJ, Schladweiler M, Richards JH, Ledbetter AD, Jarrell LT, Snow SJ, Henriquez AR, Farraj AK, Dye JA. Aspirin pre-treatment modulates ozone-induced fetal growth restriction and alterations in uterine blood flow in rats. Reprod. Toxicol. 2019; 83:63-72. doi: 10.1016/j.reprotox.2018.12.002.

Paper #12: Snow SJ, Phillips PM, Ledbetter A, Johnstone AFM., Schladweiler MC, Gordon CJ, Kodavanti UP. The influence of maternal and perinatal high-fat diet on ozone-induced pulmonary responses in offspring. J. Toxicol. Environ. Health A. 2019; 82:86-98. doi: 10.1080/15287394.2018.1564101.

Paper #13: Rogers JM. Smoking and pregnancy: Epigenetics and the developmental origins of the metabolic syndrome. Birth Defects Res. 2019; 111:1259-1269. doi: 10.1002/bdr2.1550.

Paper #14: Valdez MC, Freeborn D, Valdez JM, Johnstone AFM, Snow SJ, Tennant AH, Kodavanti UP, Kodavanti PRS. Mitochondrial bioenergetics in brain following ozone exposure in rats maintained on coconut, fish, and olive oil-rich diets. Int. J. Mol. Sci. 2019; 20:6303. doi: 10.3390/ijms20246303.

Paper #15: Valdez M, Valdez JM, Freeborn D, Johnstone AFM, Kodavanti PRS. The effects of ozone exposure and sedentary lifestyle on neuronal microglia and mitochondrial bioenergetics of female Long-Evans rats. Toxicol. Appl. Pharmacol. 2020; 408:115254. doi: 10.1016/j.taap.2020.115254.

Paper #16: Snow SJ, Broniowska K, Karoly ED, Henriquez A, Phillips PM, Ledbetter AD, Schladweiler MC, Miller CN, Gordon CJ, Kodavanti UP. Offspring susceptibility to metabolic alterations due to maternal high-fat diet and the impact of inhaled ozone used as a stressor. Sci. Rep. 2020; 10:16353. doi: 10.1038/s41598-020-73361-0.

Paper #17: Kodavanti PRS, Valdez M, Richards JE, Agina-Obu D, Phillips PM. Jarema KA, Kodavanti UP. Ozone-induced changes in oxidative stress parameters in brain regions of adult, middle-age, and senescent Brown Norway rats. Toxicol. Appl. Pharmacol. 2021; 410:115351. doi: 10.1016/j.taap.2020.115351.

Paper #18: Beasley TE, McDaniel KL, Oshiro WM, Moser VC, MacMillan DK, Herr DW. Impacts of a perinatal exposure to manganese coupled with maternal stress in rats: Maternal somatic measures and the postnatal growth and development of rat offspring. Neurotoxicol. Teratol. 2022; 90:107061. doi: 10.1016/j.ntt.2021.107061.

Paper #19: Oshiro WM, McDaniel KL, Beasley, TE, Moser V Herr DW. Impacts of a perinatal exposure to manganese coupled with maternal stress in rats: Learning, memory and attentional function in exposed offspring. Neurotoxicol. Teratol. 2022; 91:107077. doi: 10.1016/j.ntt.2022.107077.

Paper #20: McDaniel KL, Beasley TE, Oshiro WM, Huffstickler M, Moser VC, Herr DW. Impacts of a perinatal exposure to manganese coupled with maternal stress in rats: Tests of untrained behaviors. Neurotoxicol. Teratol. 2022; 91:107088. doi: 10.1016/j.ntt.2022.107088.

Appendix B

Quality Assurance

This work was conducted under the U.S. EPA Quality Assurance (QA) program to ensure data are of known and acceptable quality to support their intended use. This report summarizes research conducted under approved Quality Assurance Project Plans (QAPPs); these QAPPs include “Impact of age on the susceptibility to ozone through high fat diet or exercise in the rat”; “Cardiopulmonary and impairments from ozone exposure: Impact of omega-3 fatty acids formulations and dose”; “Interaction of chemical stressors and non-chemical factors: mechanism of hypothalamic-pituitary-adrenal axis activation, oxidative stress, neuroinflammation and mitochondrial bioenergetics”; and “Impacts of perinatal non-chemical and chemical stressors on neurodevelopment in rat offspring”. The technical aspects of this report were reviewed by two independent scientific experts from the Office of Research and Development and the Office of Chemical Safety and Pollution Prevention prior to management clearance. In addition, all findings described in this report have been published previously in highly regarded toxicological journals (see Appendix A) and subjected to rigorous peer reviews by well qualified external experts in the research fields as directed by each journal editor.

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