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Developmental Implications for Prenatal Exposure to Environmental Toxins:
Consumption Habits of Pregnant Women and Prenatal Nicotine Exposure in a
Mouse Model

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Psychology

by

Sarah Emily Santiago

August 2014

Dissertation Committee

Dr. Kelly Huffman, Chairperson

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The Dissertation of Sarah Emily Santiago is approved :

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Although this dissertation is presented as my own,
There really is no way I could've finished it alone.

My colleagues, friends, and family were always by my side,
With kindness and encouragement throughout the bumpy ride.

First to my committee, who have helped me through the years:
Who taught me, who inspired me, who sat through awkward tears.

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Glenn helped me find my voice and helped me break out of my shell,
The faith he has in science is inspiring, as well.

The lab is called a dungeon, half-jokingly i guess,
The peeling walls are windowless, and yellowish at best.

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Thanks for secret bombings with a bag of googly eyes,
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For my whole life you've been there to make sure that I'm ok.

And though these words do not in full make up for all you've done,
It is but the first token of repayment just begun.

DEDICATION

Dedicated to

Carlos Santiago and Richard Keegan

with many thanks.

“Whatever we learn has a purpose and whatever we do affects everything and everyone else, if even in the tiniest way... And it's much the same thing with knowledge, for whenever you learn something new, the whole world becomes that much richer.”

~The Princess of Pure Reason, *The Phantom Tollbooth*, Norton Juster

ABSTRACT OF THE DISSERTATION

Developmental Implications for Prenatal Exposure to Environmental Toxins:
Consumption Habits of Pregnant Women and Prenatal Nicotine Exposure in a
Mouse Model

by

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Doctor of Philosophy, Graduate Program in Psychology
University of California, Riverside, August 2014
Dr. Kelly Huffman, Chairperson

This dissertation provides a discussion of the effects of maternal consumption of environmental toxins, and will hopefully contribute to the prevention and understanding of developmental disorders and physiological deficits. Developing systems are particularly susceptible to toxic insults, and small changes *in utero* can result in long-term deficits. Chapter one of this dissertation reviews the potential teratogenicity of nicotine, alcohol, caffeine, MeHg, PCBs, BPA, and tap water contaminants, so as to characterize the current body of literature detailing the effects and implications of prenatal exposure to toxins. In chapter two, research on maternal consumption habits is presented, with an emphasis on commonly-consumed, potentially-teratogenic substances. Occurrences and frequencies of maternal intake of healthy and unhealthy foods, beverages, and medications in a population of predominantly Hispanic women

in Southern California were assessed using the Food, Beverage, and Medication Intake Questionnaire (FBMIQ). The described study reveals that a proportion of pregnant women consumed BPA, MeHg, caffeine, and alcohol at varied levels during pregnancy. The following chapters provide an in-depth analysis of the postnatal effects of a particular neuroteratogen, nicotine, which has been shown to impart various detrimental postnatal effects on exposed offspring. A CD-1 mouse model of prenatal nicotine exposure (PNE) was used to analyze aspects of the brain and neocortex that may underly some of the cognitive and behavioral phenotypes seen with PNE. Analyses included postnatal measurements of brain weight, brain widths and lengths, development of neocortical circuitry, and cortical thickness measures. Exposed mice were found to exhibit reduced brain and body weights at birth, a phenotype that recovered by postnatal day 10. No changes in neocortical circuitry or thickness in sensory and motor areas were found. PNE also resulted in persistent behavioral effects, including increased anxiety and deficits in sensorimotor integration abilities, in six month old females. Such analyses describe immediate and long-lasting postnatal effects of prenatal nicotine exposure, underscoring the importance of abstaining from nicotine during pregnancy. Hopefully, the works detailed in this dissertation will provide a foundation upon which future researchers can build a better understanding of how prenatal exposures contribute to developmental deficits.

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GENERAL INTRODUCTION

ENVIRONMENTAL TERATOGENS

Birth defects are a leading cause of infant mortality and can arise as a result of genetics, environmental and occupational exposures, physical conditions, and drug use. Defects resulting from exposure to environmental agents, termed teratogens, are potentially preventable and include neural tube defects (NTDs), physiological effects, and long lasting cognitive, psychological, and behavioral abnormalities.

One of the most infamous teratogens is diethylstilbestrol (DES), a synthetic estrogen compound reported to prevent spontaneous abortion and preterm delivery, was prescribed to pregnant women from the 1940's to the 1970's. Later studies found DES to be carcinogenic and teratogenic. The drug was eventually taken off the market, but not before millions of mothers and their children were exposed (for review, see [1]). Similarly, thalidomide was marketed to the pregnant women in the late 1950's as a "safe and harmless" antiemetic, however, the drug was later found to cause congenital malformations in newborn infants (for review, see [2]). The early 1970's witnessed a further popularization of the concept of teratology with the publication of Jones' et al. characterization of the harmful effects of alcohol on the fetus [3], and the field of teratology has since grown steadily with further identification of toxins and a better understanding of developmental biological systems.

Today, the list of substances with teratogenic properties is extensive, and includes pharmaceutical compounds like DES and thalidomide, industrial chemicals such as lead,

and illegal substances of abuse such as methamphetamines, cocaine, and heroin. Developmental deficits resulting from prenatal exposure to certain substances have physiological effects that are well documented in the literature. Less characterized are the deleterious effects of exposure to commonly consumed substances containing substances with disputed, unknown, or unpopularized degrees of teratogenicity.

The methodology used by the Food and Drug Administration (FDA) for approving substances has called into the question the validity of these categories [4]. The FDA does require clinical trials and animal studies for approval of new drugs to be used during pregnancy, but these studies usually focus on immediate adverse effects. A categorical rating system for teratogens has been developed by the FDA ranging from category A (fetal risk has not been revealed in human studies) to category x (the risks to the fetus outweigh any benefits from the substance; [5]). Such a rating system was not originally intended to be the sole source of information on teratogenic effects of a substance. Rather, the system was created to help identify possible teratogens that merit further research. Nonetheless, the rating system has been used clinically to inform the public as to the adverse effects of certain foods and medications, although its original purpose was not intended as such [6].

ASSESSING THE DEGREE OF TERATOGENICITY

Several issues arise when characterizing the extent of a given substance's teratogenicity. Firstly, it is important to note that teratogenic effects may not manifest themselves with extreme physiological phenotypes. Methylmercury in tuna, for example, is a teratogen that has been linked with a litany of cognitive deficits that are

not readily apparent at birth [7, 8]. Prenatal exposure to other substances such as polychlorinated biphenyls (PCBs; present in farmed salmon), and tobacco smoke put exposed offspring at risk for developmental disabilities, the most common of which are learning disabilities, sensory deficits, developmental delays, and cerebral palsy [9, 10].

The severity and pattern of teratogenic effects depend upon a variety of factors (Figure 1.1). Different teratogenic agents result in varying effects, and the combination of agents may potentiate or alleviate deleterious effects [11, 12]. The developmental timing of exposure is also critical. Periods in which the individual is most susceptible to toxicity vary across developmental systems as a result of temporal and regional differences in developmental processes like proliferation, migration, and differentiation. The dose of exposure must also be taken into account when characterizing teratogenicity. Toxins often exert their effects in a dose dependent manner along a continuum. Indeed, the interplay between exposure patterns and duration make it difficult to fully characterize developmental effects.

NICOTINE AS A TERATOGEN

Maternal smoking and the use of nicotine replacement therapies during pregnancy remain a concern, despite health advisories implicating nicotine as a neuroteratogen. Nicotine readily crosses the placental barrier during pregnancy and has been shown to result in numerous physiological, cognitive, and behavioral abnormalities [13-15]. Maternal smoking has been associated with pregnancy complications as well as physical abnormalities, such as low birth weights and small head circumferences in the offspring [14, 16-18].

Newborns born to smoking mothers are more excitable and show more signs of stress during the neonatal period [19]. Additionally, children whose mothers have smoked are more likely to have deficits in attention, memory, and overall cognitive function [20]. Sensorimotor deficits have also been reported in infants exposed to tobacco, particularly in responsiveness to auditory stimuli [21-23], a finding that is mirrored in research with rodents [24]. Increased incidences of attention-deficit/hyperactivity disorder (ADHD) have been documented in children whose mothers smoked during pregnancy, and smoking mothers are four times as likely to have a child with conduct disorder [25, 26]. These children also seem to be more at risk for nicotine dependence and other psychiatric disorders later on in life [15, 27]. Studies in animal models have demonstrated physiological and behavioral effects that are consistent with human studies [28].

This dissertation addresses the complex issue of teratogens in our environment and the subsequent effects on individuals and society using two discrete approaches.

The first chapter presents an in-depth scrutiny of the physiological, cognitive, and behavioral effects of developmental exposures, alongside a discussion of ensuing implications for society and for research in developmental biology. Effects of prenatal exposures to substances with addictive properties, such as nicotine, alcohol, and caffeine, are reviewed first. Developmental disorders and physiological defects resulting from exposures to these commonly consumed substances are completely preventable, yet, alleviation of such effects is complicated by the substances' addictive properties, such that pregnant women who may be aware of possible risks may have difficulties abstaining upon conception. Developmental exposures to other environmental toxins, such as methylmercury (MeHg), polychlorinated biphenyls (PCBs), Bisphenol-A (BPA),

and drinking water contaminants are consequences of bioaccumulation and environmental seepage of pollutants. Limiting exposures of these substances is made difficult by the pervasiveness of the contamination and the lack of awareness surrounding the teratogenicity and source of exposure.

The second chapter presents research investigating the consumption habits of a predominantly hispanic population in California, with frequency analyses speaking to the scope of maternal ingestion of potential teratogens. In the described study, pregnant women from Downey, California reported consumption frequencies of given foods, beverages, and medications, thus providing a window into the practical relevance of maternal environmental toxin consumption. A proportion of these women were found to have ingested potentially teratogenic substances during pregnancy. The data describing both healthy and unhealthy intake habits will hopefully discourage risky consumption practices and alleviate preventable developmental effects resultant of prenatal exposures.

A separate line of research seeks to elucidate the specific physiological and behavioral effects of prenatal environmental toxin exposures. Assays using animal models are an invaluable tool to the diagnosis, understanding, and treatment of developmental disorders resulting from prenatal exposure to environmental toxins. Thus, chapters three and four discuss research characterizing the effects of prenatal nicotine exposure (PNE) in a mouse model developed specifically for these analyses. Research concerning the effects of PNE on body weights, brain weights, brain lengths, and development of neocortical circuitry are presented in chapter three. Specifically, measures of body weight, brain weight and length were found to be reduced in newborns but recovered by postnatal day 10 (P10). Intraneocortical connections (INCs)

and thalamocortical afferents (TCAs) were also examined using anatomical tracing techniques at P0, following the supposition that gross alterations in neocortical circuitry may preclude later behavioral deficits seen in exposed children. Although no major changes in INC or TCAs were found, these analyses add to the growing body of literature by providing new information of the time course of PNE-related change in the postnatal brain.

Previous research in animal models of PNE have focused on postnatal effects during early development, adolescence, and early adulthood. Chapter four details the first study to document long-term alterations in the adult 6-month old mouse model of PNE. Mice prenatally exposed to nicotine underwent a battery of behavioral tests assessing sensory and motor function and anxiety. Measures of body weights, brain weights, and cortical thicknesses within motor, somatosensory, and visual cortices were also assessed and remain similar to controls. Though physiological and neuroanatomical effects of PNE seem to be rescued as offspring mature, data from behavioral assays in 6-month old mice suggest defects in sensory and motor function and increased anxiety persist well into adulthood.

In all, this dissertation presents a comprehensive review of developmental exposures that provides insight into the current body of literature surrounding prenatal exposures to environmental toxins. Research on maternal consumption habits is presented, with an emphasis on commonly-consumed, potentially-teratogenic substances. An in-depth analysis of the postnatal effects of a particular neuroteratogen, nicotine, follows. Effects of prenatal nicotine exposure on body weights, brain weights, INC development, cortical thicknesses of sensory and motor areas, sensorimotor function, and anxiety are discussed.

CHAPTER ONE

Implications of developmental exposures to environmental toxins

OVERVIEW

Much research has been done to characterize the harmful effects of prenatal exposure to environmental toxins, which have widespread effects on brain and body systems. The developing system in particular is more susceptible to toxic insults, and small changes in utero can result in persistent, long term deficits. Exposures can result in cognitive, psychological, and behavioral alterations, which can range from severe to subclinical. Pregnant women can control exposures to many dangerous compounds - like nicotine, alcohol, caffeine, methylmercury (MeHg), polychlorinated bisphenyls (PCBs), Bisphenol-A (BPA) - by adjusting consumption habits, however some exposures to toxins, like BPA, PCBs, and drinking water contaminants, are difficult to eliminate completely. This chapter discusses sources and effects of prenatal exposures to addictive substances and pollutants commonly found in our environments (Figure 2.1)

PRENATAL EXPOSURES TO ADDICTIVE SUBSTANCES

Developmental disorders and physiological defects resulting from exposures to nicotine, alcohol, and caffeine are entirely preventable through abstinence, however, we

continue to see a worrying proportion of women who smoke, or drink alcoholic or caffeinated beverages during pregnancy [29-31]. Such numbers may in part be explained by the addictive properties of such substances.

NICOTINE

Despite governmental warnings and anti-smoke campaigns, tobacco use remains a major public health concern. Cigarette smoke is composed of around 5,000 different compounds, including a multitude of biologically active carcinogens and toxins [32]. There is ample evidence implicating nicotine, the primary psychoactive component of tobacco, as a neuroteratogen [15]. A reported 15.1% of pregnant women smoke during pregnancy, and 2% have been found to smoke more than two packs a day [30]. Most pregnant women report the desire to quit smoking during pregnancy [33]. Nicotine replacement therapies are used as cessation aides and are often thought of as safer alternatives to cigarette smoke, despite the classification of nicotine as a Pregnancy Category C and D drug [34-36]. Thus, the impact of prenatal nicotine on public health is supposed to be far more widespread than maternal smoking prevalence statistics suggest.

Prenatal nicotine acts in the developing fetus by activating and desensitizing acetylcholine receptors (AChRs) which are differentially distributed throughout the brain early in gestation [37-39]. The appearance of precise and transient patterns of expression of AChRs are thought to play an important role in early human development. Thus, introducing exogenous sources of AChR agonists, like nicotine, has profound effects on developmental systems within the brain. Prenatal nicotine has been shown to

bind with high affinity to AChRs [40], resulting in a generally inhibitory effect on the central nervous system [41]. Researchers have found that prenatal nicotine exposure results in decreased rates of neurite outgrowth, or even retraction of neurites [42, 43]. Prenatal nicotine is thought to prematurely halt the proliferation stage, advancing the onset of differentiation (Slotkin et al., 1987c). Indeed, decreased brain weights (Santiago and Huffman, 2012), and decreased cell numbers in whole brain and certain subregions have been found in rodents (Onal et al., 2004; Slotkin et al., 1987c). This effect seems to persist into late development, as long-term reductions in cell numbers can be seen in visual and somatosensory cortices (Tizabi and Perry, 2000). Prenatal nicotine has also been shown to disrupt a number of neurotransmitter systems (Lipton and Kater 1989; Navarro, Mills et al. 1990; Seidler, Levin et al. 1992), which may account for some long-lasting behavioral effects of prenatal nicotine exposure. In particular, prenatal nicotine's targeting of the cholinergic system has been shown to result in persistent changes of dopamine-related behaviors [44, 45], review:[28]. Indirect effects of prenatal nicotine include anorexigenic, hypoxic, vascular, and placental effects that can also impact fetal development (review: [14]).

The deleterious effects of prenatal nicotine exposure on pregnancy outcomes, including increased rates of spontaneous abortions, smaller head circumferences, and lower birth weights of offspring, have been consistently documented [46-49], review: [14]. A recent study found that 5%-8% of preterm deliveries, 13%-19% of term infants with growth restriction, 5%-7% of preterm-related deaths, and 23%-34% of SIDS deaths in 2002 could be attributed to prenatal smoking [50]. Weights of children born to heavy smoking mothers have been found to be approximately 200 g lighter [51], an effect mirrored in studies using animal models [52]. IUGR is thought to be a function of

suppressed amino acid transport and diffusion of nutrients into placental circulation resultant of placental cholinergic system targeting by nicotine [47, 53].

Developmental nicotine has been implicated in disrupted glucose homeostasis [54]. Thus, it is unsurprising that prenatal nicotine has been linked with increased body weights and altered glucose metabolism later in life. review: [55]. Indeed, a recent meta-analysis found consistent associations between maternal smoking and overweight and obese offspring [56]. Sex-related effects of prenatal nicotine have also been documented. Alterations in sexual differentiation, both neural and behavioral, have been found as a result of nicotine exposure [57, 58]. In males, prenatal nicotine is thought to impact the endocrine system through targeting of the adrenal system, leading to inhibited testosterone production [59, 60]. Early onset of puberty in males exposed to cigarette smoke has also been reported [61].

There is evidence for sensorimotor deficits in children prenatally exposed to tobacco smoke, particularly in regards to auditory responses [21-23]. Lowered motor test scores on the Bayley motor scale and impairments in fine motor movements have also been reported in nicotine-exposed infants. [23]. Similarly, studies with mice have found problems with sensorimotor integration [24, 62], and sex-related differences in locomotor activity [63][24, 64]

The role of prenatal nicotine in the etiology of attention deficit/hyperactive disorder (ADHD) has been consistently documented [26, 65, 66], and children born to smoking mothers have been found to exhibit dose-dependent ADHD-like deficiencies in sustained attention, memory, response inhibition, and receptive listening [20]. Overall cognitive function and lowered IQs have also been reported [20, 65]. Nicotine-related cognitive deficits, as assessed by verbal scores on the Bayley Mental Scale can appear as

early as 13 months [23]. Similar deficits in attention, memory, and cognitive function have been found in rats and mice [67-70], although some null studies have been documented [71].

In addition to ADHD, other behavioral issues including emotional problems, conduct disorder, antisocial behaviors, and substance abuse have also been linked to maternal smoking. Exposed newborns are more excitable and show more signs of stress [19]. Studies of exposed children ranging from 3 to 5 years old have revealed increased incidences of oppositional, aggressive, and overactive behaviors [72][73]. Exposed individuals, especially girls, have increased risks for tobacco use, alcohol abuse, and drug dependence later in life, perhaps due to the effects of prenatal nicotine on catecholamine systems involved in reward circuitry [25][74]. Other reports of psychiatric problems in adolescence and adulthood include conduct disorder, depression, and antisocial behaviors in general [27][75]. Exposed males are at higher risk for criminal offenses (both violent and non-violent) involving arrests [76].

It is difficult to estimate the scope of prenatal nicotine on public health, but the widespread effects of exposure coupled with the high number of women who continue to smoke or use nicotine replacement therapies during pregnancy merits serious concern. Many of the effects are subclinical, as nicotine can have significant effects on the brain without affecting growth measures [77]. Prenatal nicotine also seems to contribute to the etiology of behavioral, psychiatric, and substance abuse problems later in life, no doubt having indirect costs in societal resources.

ALCOHOL

The study of the effects of gestational alcohol exposure began in the early 1970's as a pattern of malformations was noticed in offspring prenatally exposed to alcohol [3, 78]. FASD provides a blanket term under which all diagnoses of prenatal alcohol-related effects can be categorized; an individual may fall anywhere along this spectrum depending on exposure level and symptomology. The variation in degree and type of abnormal symptomology in FASD has made diagnosis difficult for medical professionals. Several different sets of diagnosis criteria have been developed to further identify individual who exhibit lessened effects of prenatal alcohol exposure. Fetal Alcohol Effects (FAE), Prenatal Alcohol Effects (PAE), Alcohol Related Birth Defects (ARBD), and Alcohol Related Neurodevelopmental Disorder (ARND) may be diagnosed depending on the array and degree of symptomology present. Individuals with Fetal Alcohol Syndrome (FAS) exhibit more serious symptoms often seen with higher incidences of maternal drinking but may nonetheless be diagnosed with or without confirmation of prenatal alcohol exposure provided that characteristic facial dysmorphologies, growth restriction, and CNS and neurodevelopmental abnormalities can be identified [79]. The prevalence of FASD has been difficult for researchers to ascertain, however, estimates range from 0.5 - 2 cases per 1000 live births [80]. When the full range of FASD is taken into account, prevalence of FASD in school children is thought to be as high as 2-5%[81] .

Growth restriction is apparent in children prenatally exposed to ethanol. Children are small for age (before puberty) as compared to non-exposed counterparts, with significant reductions in head circumference, weight, and height through age 10

[82, 83]. Mild facial dysmorphologies including midfacial hypoplasia, smoothened philtrum, thin upper lip, and small, widely spaced eyes with inner epicanthal folds [82] are common in children with diagnosed with FAS, a more severe form of FASD. These abnormalities develop in the first trimester, when the midline of the face is formed, and first trimester exposure has been correlated with the appearance of these features [84]. Less common effects include defects associated with skeletal, ocular, vestibular, hepatic, skin, and immune systems [85].

Ethanol-induced brain damage represents one of the most devastating and life-altering effects of maternal drinking. The brain is particularly vulnerable to the teratogenic effects of prenatal alcohol, which has been consistently shown to target the hippocampus, cerebellum, midline structures, and cortex. Ethanol triggers widespread apoptotic neurodegeneration in the developing forebrain by way of NMDAR blockade and excessive GABAA receptor activation [86] in the period of synaptogenesis [87]. It has been suggested that following prenatal ethanol exposure, NMDAR hyperactivity during withdrawal initiates a sequence of compensatory mechanisms resulting in up-regulated receptor functioning [88-90]. Such increased excitability may result in cell death [91, 92]. Prenatal ethanol exposure also seems to reduce the neurosphere capacity of postnatal neural stem progenitor cells [93]. Placentally-transferred ethanol accumulates in fetal hippocampi [94], leading to reduced overall numbers of cells in the CA1 region [95], lower dendritic spine density on pyramidal neurons, and decreased morphological plasticity after environmental enrichment [96]. In the cerebellum, prenatal ethanol has been associated with lower numbers of purkinje cells in rats and reduced vermis areas in children [97-99]. Agenesis and thinning of the corpus callosum have been widely documented [100-102] and it has been suggested that corpus callosum

damage may contribute to FASD children's difficulties with verbal learning [103]. In the cortex, prenatal alcohol targets precursors of the prospective forebrain in the ventromedial neuroepithelium, thereby delaying or inhibiting neurogenesis [104]. Indeed, researchers have found abnormal cortical thicknesses in temporal and parietal cortices [105], reduced frontal cortex sizes [106] and immature neurons in the cortical plate [107]. Alcohol has also been shown to disrupt intraneocortical connectivity [108]. In the somatosensory cortex, barrel field development is delayed and disorganized, perhaps due to a delay in thalamocortical innervation of the cortex [109-112]. Decreases in whisker and forepaw representations, along with reductions of cell numbers in somatosensory cortex, have also been observed in rodents [110, 112, 113]. Prenatal exposure to ethanol results in dendritic spine abnormalities, specifically an abnormal predominance of long, entangled spines and the lack of normal stubby and mushroom spines. Such dendritic characteristics mirror what is seen in mentally retarded children with Down's Syndrome [114].

Numerous cognitive and psychological effects have been associated with prenatal exposure to ethanol. Individuals with Fetal Alcohol Syndrome (FAS) have poor attention and concentration skills which may lay the foundation for other cognitive deficits [83], such as deficits in problem-solving and information processing and storage [115]. These difficulties can be seen throughout childhood development. 13-month old infants with FAS were found to have difficulties with information processing [116]. Exposed children have more difficulty with mathematical tasks and experience significant deficits in verbal learning [117-120]. At 14 years old, children prenatally exposed to ethanol performed lower on complex decision tasks requiring information processing [118]. Memory dysfunction is also well documented in these children [118,

120, 121] and seem to persist well into adolescence and adulthood. These effects have been mirrored in research with animal models (review: [96]). Damage to the hippocampus may contribute to some of the many behavior deficits seen in prenatal alcohol exposed offspring [122]. Streissguth and colleagues (1991) found lowered IQs in individuals with FAS, with scores ranging from 20 -100. Individuals prenatally exposed to alcohol score lower in measures related to complex attention, verbal learning, and executive function, even when accounting for IQ [123].

Sensory specific deficits have also been observed. Sensorineural hearing loss is characteristic of FAS [124]. In addition to hearing impairments, researchers have observed deficits in visual and spatial skills, as well as delayed speech and motor development [115, 124, 125]. Even low levels of ethanol exposure may have profound effects on the developing fetus. A study with infant rhesus monkeys, for example, found that low levels of alcohol exposure - equivalent to one or two drinks daily - was sufficient to produce deficits in attention and neuromotor functioning [126] without affecting birthweight or facial features.

Individuals with prenatal alcohol exposure have higher incidences of mental illnesses. Depression and anxiety disorders in particular seem to be the most prevalent [127-129]. One study found that prenatal ethanol exposure induces changes in the HPA axis that mimic what is seen in depressed or anxious individuals [130]. Behavioral problems, particularly those in the social domain, are widely reported with FASD. Studies of attachment in infancy reveal a tendency toward disorganized attachment styles in infants born to heavy social drinkers [128]. Unhealthy attachment styles in infancy may lay the foundation for social difficulties later in life. Additionally, alcohol-exposed infants are highly irritable and have feeding difficulties and sleep disturbances

[131, 132]. In early childhood, FAS children exhibit further social and behavioral problems [133, 134]. One study found that 4 year olds whose mothers drank one to five drinks per day during pregnancy were less attentive and more active than their control counterparts [135]. In adolescence and adulthood, individuals prenatally exposed to alcohol exhibit increased stubbornness and social withdrawal, and have more conduct problems such as lying, cheating, and stealing [83]. Poor socialization and communication skills are common, and FAS individuals are more likely to have problems with alcohol and drug abuse and to display antisocial behaviors [136-138]. In animal models, developmental ethanol has been associated with multiple facets of social behavior, including social investigation, aggression, and play behaviors [108, 139-141].

Rates of alcohol consumption change over time due to social and political factors that influence maternal drinking, but recent estimates of alcohol use from self-reports of pregnant women range from around six to eight percent [29, 31]. However, given the problems with recall bias and underreporting inherent in survey-mediated extraction of sensitive data, actual numbers are presumed to be higher [142].

CAFFEINE

Caffeine is a methylxanthine found in coffee, tea, soda, and chocolate. Studies have shown that caffeine consumption is common during pregnancy: Researchers investigating populations in Brazil have recently revealed caffeine consumption in nearly 100% of pregnant women, 20% of whom reported heavy consumers [143]. In the late 1980s, studies found caffeine consumptions ranging from 70% - 96% of pregnant

women, with around 7% reporting heavy usage [144, 145]. A 1999 study on US consumption revealed an average consumption of 58 mg/day (157 mg/day at the 90th percentile)[146]. More recently, survey on the consumption habits of pregnant women in Southern California found that 80.1% of pregnant women consumed caffeine during pregnancy, with almost half reporting use in all three trimesters [29]. Caffeine consumption has previously been associated with greater maternal age, less education, and being white or hispanic [145].

Caffeine primarily exerts its effects via blockade of A1 and A2 adenosine receptors [147]. Caffeine is able to cross the placental and blood-brain barrier [Cit needed] to reach the fetal brain, which has been shown to lack the principle caffeine enzyme cytochrome CYP1A2 [148] that adults use to metabolize the drug. Given the roles of developmental adenosine in neuronal inhibition [149], axonal formation [150], and neuroprotection [151], prenatal caffeine may be disrupting normal brain development through activation of adenosine receptors.

Animal studies have indicated possible deficits in neural development as a result of prenatal nicotine. In chicks, caffeine exposure has resulted in defective neural tube closures, reduced neural branching, disrupted neuronal migration, and increased susceptibility to neuronal network excitability [152][153]. Rats exposed prenatally to a dose of caffeine similar to 3-4 drinks per day have been found to exhibit altered GABA neuron migration and increased excitability in the hippocampus [154]. Prenatal caffeine may also disrupt gonadal development. Researchers have found lowered sperm counts in male rats exposed to low doses of caffeine, and low fetus viability in offspring of exposed male rats [155].

Human studies have found increased risks for fetal wakefulness and SIDS when exposed to relatively high doses of prenatal caffeine (400 and 500 mg/day, respectively; [156, 157]. Prenatal caffeine has also been implicated as a risk factor for spontaneous abortion and measures of intrauterine growth restriction (IUGR)[158], although some report conflicting results [159]. The dose-dependent association between caffeine use and increased risk for spontaneous abortion is well documented [160-162] and supported by meta-analysis [158]. However, prenatal caffeine does not seem to be related to incidences of preterm deliveries [145, 163-165]. Results of studies investigating the links between prenatal caffeine and head circumference, gestational length, and birth weights are less clear, but seem to indicate a negative impact of caffeine on intrauterine growth [145, 158, 166, 167]. Notably, this association has been found at low exposures to caffeine, although deleterious effects at low exposures may be trivial [145]. Discrepancies may stem from methodological concerns such as recall bias and lack of control for confounding factors.

There is a paucity of research investigating long-term behavioral effects of prenatal exposure to caffeine in humans, however, longitudinal studies have failed to find significant relationships between caffeine exposure and problem behavior in 5-6 year olds [168], mental and motor development in infants [169], or IQ and attention in 7 year olds [170]. In rodents, researchers have found decreases in measures of aggression [171] and deficits in certain memory tasks [154, 172].

March of Dimes recommends limiting caffeine consumption to under 200 mg/day [173]. Caffeine in excess of 300 mg/day seems to be a risk factor for IUGR and spontaneous abortions (See reviews: [174, 175]). Studies investigating the detrimental

effects of low doses of caffeine are conflicting [160, 176], however low to moderate use is not considered harmful, at least from the perspective of spontaneous abortion.

PRENATAL EXPOSURES TO POLLUTANT SUBSTANCES

Prenatal exposures to methylmercury (MeHg), polychlorinated biphenyls (PCBs), Bisphenol-A (BPA), and drinking water contaminants are consequences of pollutants infiltrating our food and water sources and our everyday environments. MeHg and PCBs bioaccumulate in the marine environment, and can be ingested by consuming certain types of fish or seafood [177, 178]. Bisphenol-A (BPA) is a xenoestrogen used in epoxy-resins and plastics. BPA is present in a multitude of commonly-used everyday items ranging from carbonless receipt paper to compact disks (CDs) [179, 180]. Contaminants of drinking water are wide-ranging, and include a group of volatile organic contaminants termed disinfection by-products (DBPs), which are formed when chlorine or chloramine react with organic matter in the water disinfection process. Limiting exposures of these substances is difficult due to the ubiquity and extent of contamination.

METHYLMERCURY

Although consumption of fish is generally thought to be beneficial during pregnancy due to high levels of fatty acids, certain kinds of fish represent a danger to the developing fetus due to the presence of methylmercury (MeHg), a widespread environmental neurotoxin with well-documented deleterious effects on the brain [181, 182]. Inorganic mercury from atmospheric depositions and other pollutants

bioaccumulate in marine food webs in the form of MeHg, which accumulates in the fetal brain more readily than in the maternal brain [177][183]. Indeed, fetal cord blood concentrations of mercury have been found to be almost twice as high as maternal blood concentrations [184]. Children are not able to metabolize mercury at the same rates as adults, therefore, low levels of tuna can result in mercury blood levels in children that exceed the health limit.

MeHg is thought to exert its developmental effects on the brain by interrupting patterns of cell fate, migration, and neural outgrowth, and cell proliferation [181][185, 186]. Animal studies of perinatal MeHg exposure have found reductions in the number layer 4 cells in the external granule cellular layer of the cerebellum [187] and long-lasting oxidative damage to the CNS as a result of MeHg-induced disruptions in the glutathione (GHS) antioxidant system [188]. Mice exposed to perinatal MeHg also exhibited sex-dependent behavioral effects on horizontal exploration and working memory in the modified T-maze, however no effects were found in motor coordination learning or reference memory [189].

A number of longitudinal studies based on populations from the Faroe Islands, the Seychelles, and New Zealand have provided insight into the behavioral and cognitive effects of prenatal MeHg.

The Faroe Islands study is based on a cohort of 1022 singleton births in a population characterized by high fish and whale consumption (Maternal hair concentrations of MeHg was found to be 4.3 ug/g [190]). Subsequent studies of the cohort found neuropsychological dysfunctions in memory, language, attention, and visuospatial and motor function that were related to maternal MhHg levels in seven-year-olds [191]. Others have found dose-dependent delays in evoked auditory and

visual potentials in exposed Faroe Island children at this age [192, 193]. Seven and 14-year-olds prenatally exposed went through neurobehavioral testing - deficits in finger tapping speed, reaction time, cued naming. deficits in motor, attention, verbal tests [194] Test scores as a result from prenatal exposure of MeHg were not affected after controlling for PCB exposure from whale blubber [195].

Studies investigating frequent seafood consumers in New Zealand have found poorer scores on full-scale IQ, language development, and gross motor skills in 6 or 7-year old children [196]

Other studies have found postnatal deficits in IQ score [197], and delays in psychomotor and mental performance in infants have also been associated with prenatal MeHg exposure [198]:

While many researchers have found associations between prenatal MeHg exposure and adverse behavioral outcomes, others have failed to find such detrimental effects. Most notably, researchers studying approximately 800 mother-infant pairs in the Seychelles have found little evidence for adverse outcomes in exposed 66 month olds [7]. Neurocognitive, language, memory, motor, perceptual-motor, and behavioural functions in exposed children at age 9 years was not adversely affected [199], nor was prenatal mercury found to be associated with negative behavioral and cognitive outcomes in a followup study of exposed children at 17 years of age [200]. Prenatal mercury via fish consumption has not been found to be associated with autism-spectrum phenotypes [201].

Efforts investigating possible impacts of prenatal MeHg are complicated by the postnatal effects of n-3 polyunsaturated fatty acids (PUFAs), also contained in fish, which have been associated with positive effects in the nervous system. Children

exposed to higher levels of PUFAs received long-lasting enhancements in visual based processes [202] and cognitive function and development [203, 204]. More recent lines of research have investigated the interplay between MeHg and PFAs [205-207]. After adjusting for PUFA n-3 status, methylmercury was found to disrupt visually evoked potentials in Faroe Island children exposed to MeHg [206]. Prenatal MeHg was found to be associated with poor development scores in exposed 2 year olds from the Seychelles, but only after adjusting for fish intake [208].

In light of the detrimental effects of prenatal MeHg, the FDA and the Environmental Protection Agency (EPA) issued an advisory concerning fish with high MeHg levels, along with a recommendation that pregnant women eat no more than six ounces of tuna per week. Mean mercury levels of tuna range from 0.128 ppm (light canned tuna) to 0.689 ppm (big-eye tuna), but can reach levels as high as 0.889 ppm for light canned tuna and 1.816 ppm for big-eye tuna [209]. Such variability in tuna samples is worrying, and recent tests performed by Consumer Reports found levels of mercury in White tuna such that eating only 2.5 ounces of any of the new samples of white tuna would cause pregnant women to exceed the daily mercury levels that the EPA considers safe [210].

Nonetheless, maternal consumption of PUFAs has proven to be developmentally beneficial to fetal development, and a diet high in certain kinds of fish that are high in PUFAs but low in MeHg are an important part of a healthy prenatal diet.

Despite the ban on production of polychlorinated biphenyls (PCBs) in most industrialized countries for more than twenty years, these industrialized chemicals remain prominent in environmental and biological systems. Lipophilic PCBs bioaccumulate in fatty tissue of marine life feeding in contaminated waters. Despite the beneficial effects seen after consuming PUFAs in fish, maternal consumption of farmed salmon nonetheless represents a dangerous risk factor for prenatal exposure to dangerous PCBs, as studies have shown significant increases in PCB level compared to their wild-type counterparts [178]. There exists a wealth of literature documenting the widespread neurobehavioral and developmental effects of prenatal exposure to PCBs (see reviews [211, 212]). PCBs represent a vast family of individual congeners, some of which are more environmentally relevant than others, and parsing out the different effects of specific congeners remains an important objective for the field. An additional concern for the prevalence of environmental and dietary PCBs is the chemicals' ability to exert its detrimental effects well after exposure has ceased. Offspring of rhesus monkey mothers who were exposed to PCBs one year prior to giving birth nonetheless experienced detrimental effects similar to offspring whose mothers consumed PCBs during pregnancy [213]. Thus, offspring could be at risk for prenatal exposure to such toxins even if pregnant women abstain from at-risk food groups during pregnancy. Indeed, a study conducted by Jacobson et al. (1984) found PCB levels in cord serum to be related to certain pregnancy outcomes, despite finding no relationship between maternal consumption and pregnancy outcomes.

Prenatal PCB exposure has been implicated in deleterious pregnancy outcome measures such as spontaneous abortions, small head circumference, reduced birth weight, and reduced fetal growth [214-216]. According to a recent meta-analysis, such fetal growth impairments have been linked to low levels of prenatal PCB exposure [216]. Moderate yet clinically-relevant levels of exposure were found to be associated with a 160-190 gram weight decrease in exposed newborns as compared to their non-exposed counterparts [214]. Additionally, newborns whose mothers had consumed higher amounts of contaminated fish were found to have abnormally weak reflexes, immature autonomic responses, greater motor immaturity, and over-reactivity to stimulation [212, 217-220]. Dose dependent decreases in scores on Fagan's tests of infant intelligence and visual recognition have also been documented [221, 222].

Cognitive deficits in preschool aged children exposed prenatally to PCBs have been consistently documented [212, 220, 223, 224]. These children exhibit impairments in short-term memory function on verbal and quantitative tests [224]. Additionally, researchers have found that children prenatally exposed to PCBs are at risk for developing behavioral problems and poor response inhibition [225, 226]. Although some researchers have reported a functional recovery of certain measures of cognitive impairment by 54 months [227], others have reported continuing problems with cognitive functioning in older children [225, 228], see review[212]).

Literature cataloging the developmental effects of PCBs on animal models is extensive, Although there seems to be widespread variation in dosing concentrations, routes of administration, and PCB chemicals used, research using animal models has largely corroborated the cognitive and behavioral impairments in human studies. For instance, research using animal models has unveiled spatial learning and memory

deficits, and impaired LTP associated with PCB exposure [229, 230]. Disrupted neurological function has also been reported. For example, in mice, severely affected individuals are termed “spinners” and display stereotypical head bobbling, hyperactivity, and rotational movements [231, 232]. Non-spinners also exhibited hyperactive behaviors and had difficulties with certain motor tasks [232]. Such hyperactivity may be a result of lower levels of dopamine and reduced dopamine receptor binding sites in the caudate nucleus [230, 233, 234]. Developmental PCB exposure has also been found to deplete certain thyroid hormones, and affect numerous estrogenic, androgenic, and anti-androgenic systems (for review, see [211]). Sex-specific effects on apoptosis in the anteroventral periventricular nucleus (AVPV) and altered sexual differentiation of the preoptic area (POA) have been found in neonatal rats [235]. Thus, it is not surprising that prenatal PCB exposure has resulted in changes to mating behaviors in female rats [236].

In all, exposure to developmental PCBs has been revealed to be particularly worrisome, due in part to the wide-ranging developmental effects which include poor pregnancy outcomes, changes in neurobiological and hormone function, and subsequent impairments in developmental, cognitive, behavioral, and reproductive function. Furthermore, accumulation of PCBs in fatty tissue presupposes the major contribution of past contaminated fish consumption in prenatal PCB exposure. Thus, careful avoidance of PCB contaminants after conceiving does not necessarily prevent deleterious effects of prenatal PCB exposure.

BISPHENOL-A

Bisphenol A (BPA) was first produced in the late 19th century, but the estrogenic characteristics of BPA weren't identified until the 1930s during a search for a commercially distributable synthetic estrogenic compound (review: [237]). Though its candidacy was usurped by the potently teratogenic DES -- a substance that was subsequently prescribed to millions of women, leading to widespread carcinogenic and teratogenic exposure [1]-- BPA re-emerged in the 1950s as a building block for epoxy resins [237] and continues to be produced today at an estimated rate of 8 billion pounds per year [238]. The recent vilification of BPA as a developmental toxin has led to a ban by the FDA on the use of BPA in sippy cups and baby bottles [239]. Nonetheless, BPA remains one of the more ubiquitous environmental toxins in our environment, and can be found lurking in such commonly-used everyday items as reusable water bottles, carbonless paper, CDs, dental sealants, electrical equipment, and hospital equipment [179, 180].

The ubiquity of BPA in plastics and canned foods coupled with the routine presence of the compound in urine samples suggests that BPA consumption is routine [239]. Particularly high levels of BPA were found in fetal cord serum, and estimates for BPA exposures in infants are also presumed to be very high [239]. A 2009 study found that neonates in intensive care units had abnormally high BPA levels, a result, the authors suggest, of medical supply products used [240]. Such early exposures are worrying given the exceptional vulnerability of fetuses and neonates to BPA toxicity [241].

A known endocrine disrupter and xenoestrogen, BPA exerts its effects by binding to estrogen receptors [242], and by producing estrogenic metabolites, some of which may be even more potent than itself [243]. The fetal toxicity of BPA is underscored by its failure to readily bind to sequestering plasma proteins that normally protect developing tissue from excessive estrogen, thus, BPA may have a stronger effect on the fetus because it sidesteps key protective mechanisms in utero. Developmental BPA is also thought to disrupt other systems: it can bind to glucocorticoid [244] and androgen receptors [245], and has been shown to disrupt dopamine systems [246] and thyroid hormone systems [247]. Researchers have also found epigenetic modifications resultant from developmental BPA [248, 249].

Although BPA does not seem to result in deleterious pregnancy outcomes related to birth weight and gestational length [250], developmental BPA does seem to have an impact on body weights later in life. Given BPA's actions on the glucocorticoid and thyroid hormone pathways [244, 247], it is not surprising that developmental BPA was found to result in increased rat body weights observable into adulthood [251]. Researchers have also found heightened expression of adipogenic gene expression in rats exposed to BPA [252].

In animal models, numerous carcinogenic and reproductive effects have been found to be associated with developmental BPA exposure [253]. In males, developmental BPA has resulted in decreased testis weight [254], diminished spermatogenesis [255, 256], and increased adult prostate weights [257]. Females exposed to developmental BPA have been found to exhibit altered mammary gland histoarchitecture, increased susceptibility to mammary cancer, and masculinization of sexually dimorphic brain area APVP, even at low doses [258-260]. Effects of BPA on reproduction include changes in rat sexual

behavior, as evidenced by increased female receptivity as well as male defensiveness and aggressiveness [254, 261]. In humans, BPA exposure has been associated with advancement of puberty [262, 263], and BPA associations with male aggressiveness has also been documented [264].

Other behavioral effects resultant of developmental BPA include hippocampal ACh-related memory impairment in mice [265], and dopamine-related hyperactivity in 4-5 week old rats. Developmental BPA may also play a role in behavioral responses to cocaine and amphetamine exposure by mimicking the modulatory role of estrogen on the reward system [266, 267]. Indeed, mice exposed to developmental BPA were found to be more sensitive to methamphetamine-induced behavioral responses [246]. In humans, studies in very young children have linked prenatal BPA with sex-specific behavioral problems, specifically emotional reactivity and aggression, on the CBCL (child behavior checklist) [264]. Similarly, developmental BPA has been found to be associated with externalizing behaviors in two year old infants [268], and with more anxious and depressed behaviors and poorer emotional control in 3 year olds [269].

Unfortunately, prenatal exposure to BPA is difficult to measure with survey instrumentation, and may be difficult to avoid, as exposure can occur as a result of contact with normal, everyday items. Particular concerns for exposure in pregnant women include consumption of canned foods, as leeching of BPA from resins coating metal food cans has been well-demonstrated [270, 271]. A recent report found almost three quarters of women sampled consumed canned foods during pregnancy, and almost 12% consumed canned foods more than four times per week [29]. Consistent handling of carbonless paper also represents a risk to pregnant women. Exceptionally

high levels of BPA were found in pregnant cashiers, presumably from contact with receipt paper [272].

Also worrisome are the number of studies reporting effects with doses of BPA lower than the EPA's reference dose of 50 µg/kg/day [273], although the extent to which BPA exerts deleterious effects remain contested [274, 275]. Nonetheless, the amount of literature implicating BPA as a potential toxin to developing organisms is vast and generally supported by clinically relevant dosage models [239].

CONTAMINANTS IN TAP WATER

Tap water from public systems contains a plethora of microbiological, radioactive, inorganic, and organic contaminants that pose a threat to public health. Such contaminants include arsenic, lead, chromium, carbon tetrachloride, and radium. Among the most studied are a group of volatile organic contaminants termed disinfection by-products (DBPs), which include both trihalomethanes (THMs) and haloacetic acids (HAAs), and arise when chlorine or chloramine react with organic matter in the water disinfection process. Total concentrations of major THMs - chloroform, bromoform, bromodichloromethane, and dibromochloromethane - are regulated by the EPA, who caution against levels exceeding 80 ppb (parts per billion) in treated water [276]. In the US, 84% of households get water from public systems, subjecting pregnant women to toxins via ingestion or dermal exposure Bureau [277].

For the most part, current knowledge concerning the teratogenicity of water contaminants has been ascertained from epidemiological studies investigating the link between exposure and adverse outcomes during fetal development or at birth. Such studies have implicated prenatal THM exposure as a risk factor for fetal death.

Researchers have found that THM and arsenic exposure is correlated with increased risk for spontaneous abortions [278-280], as well as with an increased risk of stillbirths [280, 281] at an exposure dose that is clinically relevant (with exposure levels of about half the maximum contaminant level set by the EPA; [282]). Although some studies have failed to find strong associations between exposure and fetal death, researchers routinely cite inadequate exposure assessment methodology as a confounding factor, which may account for the apparent lack of results [283, 284]. Later research revealed the detrimental effects of DBPs was specific to THM exposure, rather than exposure to HAAs [285].

Contaminant exposure has also been implicated in increased risk for low birth weights, preterm births, intrauterine growth restriction (IUGR) and small for gestational age (SGA) infants. Although there is a general consensus regarding the association between exposure and IUGR/ SGA [284, 286, 287], there is less agreement about the link to low birth weights and preterm births, [284, 287-290]. Increased risks for NTDs and cardiac defects have also been reported [284, 288].

While there has been some research evaluating the link between drinking water contaminants and adverse outcomes at birth, research investigating causal relationships is lacking. Also, the long-term psychological, cognitive, and behavioral teratogenicity of tap water contaminants has been woefully under-researched, although links to autism and risky behaviors later in life have been suggested [291, 292].

The toxicological profile of water varies from region to region, and databases of water samples are maintained in compliance with federal regulations, facilitating correlational cohort studies investigating such birth outcomes as SGA and fetal death, for which states also keep records. Complications arise, however, with methodological

difficulties concerning assessment of individualized exposure levels, which vary with route of administration (ingestion versus dermal exposure), distance from the water distribution centers (areas further from disinfection centers have higher levels of THMs; [280], the season of the year in which the exposure took place, maternal geographical relocation, and of course, the toxicological profile of the water. Additionally, few studies have investigated consumption practices of contaminated drinking water, and those that have are subject to recall biases and inaccuracies. Such considerations may help to explain the lack of consensus for many outcomes. It is clear, however, that THMs present a danger to the fetus, with prenatal exposure resulting in SGA, IUGR, fetal death, and possibly low birth weights, NTDs and cardiac problems.

DISCUSSION

In recent decades, major public health concern over infectious disease has waned in the wake of detrimental effects from chronic diseases and conditions. According to the National Center for Health Statistics, the top four leading causes of US public death stem from chronic disease [293], the causes of which are often multifactorial in nature. Thus, the integration of research from varying fields investigating the possible contributions to chronic disease is becoming increasingly important. Prenatal exposures to environmental toxins lead to changes in the brain and body that lead to physiological, cognitive, and behavioral problems later in life (Figure 2.2).

The developing fetus in particular is more susceptible to insult from environmental influences, and small exposures in the fetal environment can translate into increased risk for chronic disease later in life. Research into the fetal origins of

chronic disease was spearheaded by David Barker's examination of the relationship between impaired fetal growth and the epidemic of coronary heart disease [294, 295], and the increased vulnerability of developing systems to toxins has been corroborated by many researchers who have found various neurobehavioral, carcinogenic, respiratory, and physiological effects as a result of prenatal exposures [296-298]. Fetuses may also have increased risk for detrimental effects due to increased chemical to body weight exposures and in some cases, increased accumulation in cord blood levels of toxins [184].

Additionally, early development is characterized by reprogramming of methylation patterns and high rates of DNA synthesis [299]. Thus, exposed offspring are highly susceptible to long-term detrimental effects originating from dysregulations of the epigenome following conception (fig. 4.1). Research documenting prenatal exposure-induced heritable changes in genotype or phenotype has primarily focused on alterations in DNA methylation, histones, and non-coding RNA expression (for review, see: [300]). Small changes in DNA methylation early in development can have a significant increase in risk for disease later in life [301].

Developmental DES exposure alters methylation in mice [302], and has been shown to result in carcinogenic effects not only in exposed offspring, but in the subsequent generation as well [303], presumably through epigenetic mechanisms. Similarly, prenatal alcohol exposure may impart some negative effects through epigenetic disruptions [304], as chronic exposure to alcohol has been shown disrupt one-carbon metabolism, thereby affecting DNA methylation and histone function [305], and leading to a decreased choline to creatin ratios [306]. Hypermethylation and histone changes in mice perinatally exposed to MeHg have been linked with suppression of

hippocampal BDNF expression [307]. Alterations in methylation patterns have also been documented for nicotine, tobacco smoke, trihalomethanes, and BPA (reviewed in [300, 308, 309]). Though less thoroughly characterized, transgenerational effects have been found with exposures to nicotine [310] and BPA [311]. Thus, prenatal exposure to certain toxins is especially worrying because of the potential to impart long lasting effects on multiple generations via epigenetic changes in the germline.

Characterization of exposure effects is complicated by a lack of understanding of combinatorial actions resulting from exposures of multiple compounds (Figure 1.1). Exposure to one drug may alleviate, potentiate, or interact with the effects of another. For instance, researchers have revealed that alcohol, caffeine, and tobacco are often used concurrently [11], and one study showed that prenatal caffeine can magnify detrimental effects of prenatal alcohol on birth weight, litter size and postnatal mortality in rats [312]. Counteractive effects have been found with prenatal nicotine and marijuana use: although prenatal nicotine has been shown to result in IUGR, concurrent use of marijuana has been shown to result in higher birth weights [176]. Additionally, consumption of omega-3 fatty acids and MeHg in fish has been shown to result in a neutralization of the beneficial effects of omega-3s and the detrimental effects of MeHg [12]. It is also quite possible that combinatorial effects may also be seen with exposures to PCBs, as the PCB family contains an extensive number of dangerous compounds that may interact with each other. Data on individual and combinatorial effects of environmentally relevant toxins will illuminate the mechanisms by which multifactorial neurological, physiological, and behavioral deficits arise.

Less overt changes can be seen in the developing brain. For many of these toxins, the mechanism of action involves targeting of receptor systems whose precision of

interplay with other biological systems are integral to the proper development of physiology and behavior. Prenatal exposure to environmental toxins can disrupt neurotransmitter systems, cell number in specific brain areas, interneocortical connectivity, and neurotransmitter systems, subsequently leading to cognitive, behavioral, and psychological problems. For example, PCB targeting of sexually dimorphic brain areas early in life may lead to dysfunctions in reproductive behaviors in life [235, 236]. BPA-induced suppression of hippocampal ACh is associated with memory problems in mice [265]. Similarly, prenatal exposure to alcohol results in neuron number and spine density reductions in the hippocampus in addition to spatial memory and learning deficits later in life (reviewed: [96]).

Research investigating longitudinal effects has been plagued by methodology complications which range from problems with recall bias in exposure assessment to difficulties in parsing exposure effects from a multitude of confounding variables. Nonetheless, continuing exploration into the subtle longitudinal effects of these toxins is paramount. Firstly, ongoing research precludes the misguided perception that healthy pregnancy outcomes confirm non-toxicity. The lack of physical abnormalities at birth may be misinterpreted as permission for future maternal consumption - a concern, given that other research may exist demonstrating the long term neural or behavioral abnormalities. The public would benefit from dissemination of knowledge about long-term effects of teratogenic substances, which often have subtle effects that manifest later in development. Commonalities in long-term effects of exposure to varying environmental toxins have arisen. For example, longitudinal effects of developmental MeHg, PCB, BPA, nicotine, and alcohol all include cognitive deficits which may span

memory, attention, IQ, decision making, and overall cognitive ability [65, 194, 196, 230, 265]. Evidence for long term effects on social behaviors and psychiatric disorders are also well documented for many of these toxins, perhaps due to the targeting of neurotransmitter and hormone systems early in development [44, 246]. Additionally, prenatal exposures have been linked to obesity-related issues later in life [55, 251]. Such long-term effects have the potential to be a significant drain on societal, medical, and educational resources in the form of visits to physicians, loss of productivity, prescription drugs, and special education services [313]. Even subclinical effects of prenatal exposures can have profound effects on the population by reducing the overall intelligence and productivity of a society [298].

Controlling exposure levels for environmental toxins is therefore a very worthwhile endeavor. The most straightforward method involves changes to the behaviors of pregnant women, as detrimental effects resulting from prenatal exposures to caffeine, nicotine, alcohol, and MeHg are, for the most part, preventable. Alleviation of effects from addictive substances such as alcohol and nicotine might benefit from programs and cessation aids. Increasing awareness of toxicant levels of BPA in canned foods, PCBs in farmed salmon, and MeHg in tuna, along with educational interventions detailing resultant detrimental effects would also aid in controlling exposure levels. Unfortunately, many women remain uninformed as to the potential dangers of consuming certain foods and beverages during pregnancy. Although the exact reasons for this are difficult to assess, the gap between science and practice could be accounted for by a paucity of research on less popularized substances. Because regulation of prenatal consumption demands a very high level of evidence of teratogenicity, little

researched substances often go unregulated and health care professionals assume they are healthy. Perceived risk is complicated by popularized research exonerating the toxicity of a given compound in adults, even though developing systems may be more susceptible. The problem could also lie in reduced access to healthcare, or time constraints in prenatal consultations.

Controlling exposures of other environmental toxins, such as PCBs, BPA, and contaminants in drinking water is problematic and may require large-scale infrastructural modifications. PCBs represent a unique danger because these compounds accumulate in human tissue and may exert effects long after abstention [213]. Indeed, one study found that while consumption of PCBs were not correlated with low birth weights, cord serum PCB levels were, providing evidence for residual effects of previously consumed PCBs [214]. The ubiquity of BPA in commonly used, every-day items also renders controlling exposures through change of maternal consumption behaviors somewhat ineffective. Although pregnant women can reduce exposures by limiting consumption of foods and beverages in cans and plastic bottles, avoiding other plastics in daily environments is nearly impossible [239]. Similarly, contaminants in tap water are difficult to avoid, as exposure can occur through dermal exposure to water from public systems and ingestion from restaurants or improperly filtered water.

Fortunately, a number of other consumables can increase the likelihood of healthy outcomes. Methyl groups in prenatal vitamins have been shown to reduce risk for neural tube defects and autism via contribution to one-carbon methylation pathways [314] [315] [316]. Given that prenatal alcohol disrupts one-carbon methylation, it's not surprising that augmenting choline during gestation has been shown to attenuate effects of prenatal alcohol exposure, namely, birth, brain weight, and many behavioral

measures [317, 318]. Pregnant women should also be encouraged to maintain high consumption levels of PUFA-containing fish, as these fatty acids may counteract some detrimental effects of MeHg and have been shown to have a beneficial effect on developing offspring [319].

CHAPTER TWO

Consumption habits of pregnant women: a survey of predominantly hispanic women in California

OVERVIEW

Healthy post-pregnancy outcomes are contingent upon an informed regimen of prenatal care encouraging healthy maternal consumption habits. In this chapter, potential implications for unhealthy prenatal dietary choices is discussed. Studies in developmental neuroscience have shown that certain substances may cause teratogenic effects on the fetus when ingested by the mother during pregnancy.

It is important to appraise consumption habits in order to alleviate future effects of developmental issues. Aspects of maternal intake of food, drink, and medication in a population of predominantly Hispanic women in Southern California were assessed by way of the Food, Beverage, and Medication Intake Questionnaire (FBMIQ), which was developed to measure common practices of maternal consumption during pregnancy. The FBMIQ was administered to English and Spanish speaking pregnant and recently pregnant (36 weeks pregnant - 8 weeks post-partum) women over the age of 18 who were receiving care from a private medical group in Downey CA. Consumption habits of healthy foods and beverages, unhealthy foods, unhealthy beverages, and medication are characterized. Data indicate widespread consumption of fresh fruit, meats, milk and juice and indicate most women used prenatal vitamin supplements. Those potentially

harmful substances included in our study were Bisphenol-A (BPA), methylmercury, caffeine, alcohol and certain medications. Results show that a proportion of the women surveyed in our study consumed BPA, methylmercury, caffeine, alcohol, and certain medications at varied levels during pregnancy. This represents an interesting finding and suggests a disconnect between scientific data and general recommendations provided to pregnant mothers by obstetricians. The results of our study demonstrate that a proportion of pregnant women consume substances that are potentially teratogenic and may impact the health and well being of the offspring.

BACKGROUND INFORMATION

Post-partum outcomes for mother and child are linked to maternal consumption habits during pregnancy [320, 321]. Thus, it is imperative that pregnant women be informed of the risks and benefits of certain dietary practices. This is not a simple task, as many food, beverages and medications carry unknown risk [322]. Additionally, most primary care physicians and obstetricians are not aware of the dietary and over-the-counter medication intake practices of their patients and thus lack the information needed to help guide them. Assessment of common practices of food, drink, and medication intake during pregnancy informs the direction of preventative practice and interventions benefiting populations of pregnant women and their offspring. As prenatal exposure to certain environmental toxins, many of which are found in common foods and beverages, can lead to developmental deficits and malformations, common consumption practices must be appraised in current samples of pregnant women. These types of behavioral assessments play a critical role in prevention of adverse

developmental outcomes that are potentially linked to a mother's intake of unhealthy substances in food, beverages and medications throughout pregnancy.

Studies investigating the impact of prenatal diet on the offspring date back to the 1920's [323], and many deleterious effects of poor diet have been reported in the literature. Reduced maternal nutrition has been associated with hypertension and altered nephrogenesis in the offspring [324, 325]. Additionally, prenatal deficiencies in vitamins D and E have been associated with increased incidences of respiratory difficulties, including wheezing and asthma, in the offspring [326], and maternal vitamin D deficiencies have been related to observed hyper-locomotion in the adult rat [327]. Maternal diets high in omega-3 fatty acids may reduce sensitivity to allergies in the offspring [328], whereas methyl-donor group (vitamin B12, folic acid, and choline) supplementation during gestation is associated with increased risk of allergic inflammation in the offspring [329]. Increased cation consumption (magnesium, potassium, and calcium) was inversely related to diastolic pressure in infants [330]. These studies demonstrate that a healthy balance of nutrients play an important role in normal developmental biology.

Prenatal exposure to environmental toxins profoundly affects the developmental biology of the fetus. It is believed that exposure to toxins during the prenatal period induces developmental changes in the brain that lead to abnormal cognitive and behavioral phenotypes. Prenatal exposures to illegal drugs of abuse, such as cocaine, heroin, and methamphetamines, have been shown to impact both the developing brain and behavior [331-333]. Similarly, exposures to legal substances that are more commonly used during pregnancy, such as alcohol and nicotine, have been shown to have both short- and long-term effects on the developing baby [14, 52, 108, 334]. Our

developmental neurobiology laboratory has created two separate animal models of prenatal alcohol and nicotine exposure with compelling results [52, 108] suggesting their high-risk status during pregnancy. It is common practice for obstetricians to warn pregnant women of the dangers of illegal drug use while pregnant, but there is less agreement in the field as to the exact recommendation for nicotine, caffeine, alcohol and many over-the-counter (OTC) medications and prescribed drugs [335-337]. Specifically, obstetricians vary greatly in their recommendations of alcohol consumption during pregnancy with some noting that occasional light use is permitted and safe, while others suggest complete abstinence [338, 339]. There are no strict guidelines in obstetrics as to whether drugs labeled by the Food and Drug Administration (FDA) as Pregnancy Category C (found to generate birth defects in animal models) are to be prescribed. Obstetricians often disagree on safe levels of caffeine consumption during pregnancy, despite its link to negative behavioral effects in the offspring [168]. Some physicians may not be aware of how pervasive intake of legal substances such as alcohol, nicotine, caffeine or OTC medication may be and, thus, fail to inform the patient of details regarding use of these common, legal substances. Behavioral assessments of consumption of these potential teratogens, in populations of pregnant woman, are thus imperative to effective preventative obstetric practice.

In addition to illegal and legal drugs, significant sources of dietary impact on the developing fetus are teratogenic substances present in unassuming ordinary foods. A wealth of new information regarding the health of our nation's food supply has been a subject of great recent interest and emerging studies have illuminated the harmful effects of methylmercury [8] and PCBs (polychlorinated biphenyls) [212], two common neurotoxic seafood contaminants present in high levels in tuna and farm-raised salmon,

in offspring exposed to the toxins during gestation. Additionally, Bisphenol-A (BPA) is a potentially teratogenic xenoestrogenic monomer found in the lining of cans used for food storage. Consequences of prenatal exposure to BPAs include behavioral and reproductive abnormalities [255, 268], increases in susceptibility for later developing mammary cancer [259], and alterations in reproductive systems. Understanding the rate at which pregnant woman consume certain food items is particularly important, as the list of foods that contain potential or known teratogens is growing. Researchers must strive to identify if pregnant woman are consuming toxins, and if so, preventative instructional measures should be taken by prenatal care providers to teach pregnant woman about the dangers of foods containing substances like mercury, PCBs, or BPA.

The Hispanic population is at risk for higher fat, sodium and caloric intakes, particularly from dairy foods [340, 341]. Additionally, acculturation in Hispanic populations has been thought to result in diets higher in fat and lower in fiber, with significantly lower intakes of protein, calcium, vitamin A, vitamin C, and folic acid [342]. Risk factors such as low socioeconomic status (SES), acculturation to US diet, and limited access to health care in Hispanic populations increase the importance of studying this population in respect to potential adverse outcomes of poor prenatal diet [343].

This chapter describes our findings in a sample of pregnant or perinatal women in Downey, California. We used anonymous survey measures to determine what foods, beverages, and medications pregnant women were consuming throughout gestation. Our results highlight the pervasive consumption of teratogens such as mercury and BPA in our sample. We hope that information gleaned from our study will help inform

prenatal care providers of the dangers and nature of certain consumptive habits among pregnant women.

MATERIALS AND METHODS

Questionnaire:

The Food, Beverage, and Medication Intake Questionnaire (FBMIQ) survey was designed to collect data on the prevalence and pattern of maternal consumption habits. The FBMIQ takes approximately five minutes to complete and contains questions concerning demographic information (household income, ethnicity, and age) how often and during what times during the pregnancy subjects (1) ate certain foods (e.g. fresh fruit, meat, and fast foods), (2) drank certain beverages (e.g. regular coffee, beer, and juice), (3) ingested prescription and over-the-counter medications during pregnancy. The FBMIQ is not a complete dietary assessment; it is designed to be a short survey reflective of consumption of certain commonly consumed, relevant items. Portion size and number of servings consumed were not assessed. Based on our laboratory's work in a Fetal Alcohol Spectrum Disorders (FASD) mouse model, we were initially interested in patients' alcohol consumption during pregnancy. However, we comprised a list of potential or known teratogens as well as healthy and unhealthy practices and used a combination of these to create the survey. Non-threatening wording and reverse coding were used to maximize subject well-being and ensure accuracy.

Participants and procedure:

Pregnant and recently pregnant women (36 weeks pregnant - 8 weeks postpartum) over the age of 18 were invited to complete the FBMIQ upon check in at the

reception desk of obstetric and gynecology (OBGYN) offices of a private medical group in Downey, CA between December, 2011 and December, 2012. Participants received the FBMIQ from receptionists and were informed that it was an optional nutritional survey about the habits of pregnant women. After completing the survey, participants placed completed consent forms and surveys in a blank sealable envelope provided to ensure confidentiality and alleviate concerns about anonymity. Surveys in sealed envelopes were then returned to the front desk where the receptionists collected the completed surveys. Frequency statistics for the data were presented in tables and differences between groups of age, education, and income were analyzed using Pearson correlation coefficients and non-parametric Kruskal-Wallis Tests. All statistical analyses were obtained using SPSS version 17.0. This study was conducted in strict accordance with the protocol guidelines approved by the Human Research Review Board at the University of Riverside, CA.

RESULTS

Demographics and participant information:

Participants were English and Spanish speaking pregnant or recently pregnant women (36 weeks pregnant - 8 weeks post-partum) over the age of 18 who were receiving Obstetric care from a private medical group in of Downey CA. A total of 200 pregnant or recently pregnant women completed the FBMIQ. Most women (89.1%) were aged 35 or younger, with the greatest percentage (34%) of participants belonging to the 30-35 age group. The subject population was predominantly Hispanic (87.4%). The remainder self-identified as 4.7% African-American, 4.2% Asian/Pacific Islander, 2.1%

White, and 0.5% Middle-eastern. Almost all of the women (95.8%) had obtained a high school degree. Women were most likely to have completed some college without obtaining a final degree (41.9% of respondents). Less than a third of the women (26.2%) possessed a college or post-graduate degree. Women were most likely to have a yearly income of \$25,000 or less (35.1%). Over two thirds (69%) of women sampled had a yearly income of 50 k or less. Thirteen point four percent had an income of over \$75,000 (Table 1.1).

Month of pregnancy confirmation:

Nearly half (50.5%) of the women sampled confirmed their pregnancies in the first month of gestation. That number rose to 88% by the second month. The vast majority of women (97.9%) confirmed pregnancy sometime in the first trimester (data not shown).

Foods beverages and medicinal intake during pregnancy

Participants were asked about food, beverage, or medication intake during their pregnancies. Specifically, they were asked to identify types of foods, beverages or medications consumed and were given the option to write in choices that were not explicitly listed in the survey (See Tables 1.2, 1.3, 1.4). Participants were also asked to report the frequency and trimester specificity of consumption (Table 1.5).

Food consumption habits:

Participants were asked whether they ate chicken, beef, or pork during their pregnancies (fish consumption patterns were assessed in a different survey section, and thus fish was excluded from the meat category). Almost all of the women sampled (99.5%) ate meat some time during their pregnancies and almost two-thirds (66.1%) ate

meat during all three trimesters (Table 1.3, Table 1.5). Chicken was the most frequently reported meat consumed (98.5%), followed by beef (84%), then pork (52%). Most women (81.9%) consumed meat at least once a week, with 30.4% consuming meat at least four times a week (Figure 3.1, Table 1.2, Table 1.5).

Most participants (73.9%) consumed fish during pregnancy. The most commonly consumed type of fish was tuna (52%), followed by tilapia (34.2%) and salmon (25.5%). Less than a quarter of the women (19.9%) also reported eating other kinds of fish or shellfish, with shrimp being the most frequently written in option. Most women ate fish less than once a week (80%), and nearly a third of women (31.2%) ate fish during all three trimesters (Figure 3.1, Table 1.2, Table 1.5).

All women reported eating fresh fruit during their pregnancies. Bananas were the most commonly eaten fruit (95.4%), followed by oranges (88.8%), and apples (88.3%) (Figure 3.1, Table 1.2). Women also reported eating other fruits such as strawberries, pears, watermelon, and grapes. Two thirds (65.8%) of women ate fruit at least four times a week. Although the majority of women (77%) reported consuming fruit during all three trimesters of their pregnancies, only a third of the women (31.1%) ate the recommended amount of more than one serving of fruit per day (Table 1.5).

The majority of participants (73.9%) reported consuming canned foods during their pregnancies, with 11.9% reporting consumption frequencies of four or more instances a week. Nearly half of sampled women (48.9%) reported eating canned foods during all three trimesters of their pregnancies (Table 1.5). Canned fruits/vegetables and soup were most commonly consumed (52.3% and 41.6% of women, respectively) followed by canned tuna (41.6%) (Figure 3.2; Table 1.2).

Almost all women (97.5%) reported eating high-sugar desserts during their pregnancies. Ice cream was the favored dessert: 82.7% reported eating ice cream. Additionally, 70.1% reported eating baked desserts, 65% reported eating chocolate, and 3.6% reported eating other desserts, such as candy or frozen dessert beverages during their pregnancies. Most women (76.2%) consumed desserts between one time per month and three times per week. Over half (53.2%) reported eating sugary desserts throughout their pregnancies (Figure 3.2, Table 1.2, Table 1.5).

Nearly all of the women (96%) reported eating fast foods during their pregnancies, with burgers being the most commonly consumed item (85.2%), followed by french fries (77.9%), chicken products (53.6%), and other fast foods such as Mexican fast foods and chicken salads. Consumption patterns for fast food intake were varied: 19.1% reported only eating fast foods 1-3 times during their entire pregnancies, 47.5% reported eating fast foods 1-3 times per month, and 25.7% reported eating fast foods 1-3 times per week. Only 5.5% of women reported eating fast food more than four times per week during their pregnancies. Forty-three percent of women reported eating fast foods during all three trimesters (Figure 3.2, Table 1.2, Table 1.5).

Beverage consumption habits:

All women reported drinking water, with 96% reporting consumption of bottled water, 36.9% consuming home-filtered water, and 12.1% consuming tap water during their pregnancies (Figure 3.1 and 2.2, Table 1.3).

Most women (95.4%) drank milk during their pregnancies. Of those women, 79% drank low-fat milk, 20.9% drank whole milk, 6.7% drank organic milk, 3.9% drank skim milk, and 8.6% drank “other” milk, with 3.6% identifying “other” as soy milk. Two-

thirds of women (66.6%) drank milk at least 4 times per week. Most (71.4%) drank milk during all three trimesters. (Figure 3.1, Tables 1.3, Table 1.5).

A total of 94.9% of women reported drinking juice during their pregnancies. Orange juice was the most commonly consumed juice (76.8%) followed by apple juice (69.1%), juice blends (41.2%) and other juices (12.9%) such as cranberry or pineapple juice. Most women (85.5%) reported drinking juice at least once per week, and over two thirds (68%) reported drinking juice throughout their pregnancies (Figure 3.1, Table 1.3, Table 1.5).

Participants were asked to report whether they had consumed regular coffee, regular tea, colas, or decaffeinated beverages during their pregnancies. The majority of women sampled (80.1%) had consumed caffeinated beverages during their pregnancies (Figure 3.3, Table 1.3). Colas were the most popular caffeinated beverage (60.2% of all pregnant women), followed by coffee (45.5%) and tea (29.8%). Forty point four percent of women who reported drinking caffeinated beverages did so throughout their pregnancies, while nearly a fifth (18.6%) only consumed caffeine during the mid- and later parts of their pregnancies (Figure 3.3, Table 1.3, Table 1.5).

Out of 190 women who responded to any questions about alcohol usage, 5.8% reported drinking alcohol sometime during their pregnancies (Figure 3.3, Table 1.3). When asked about the type of alcohol consumed, these women reported drinking beer, wine, or champagne. Consumption of mixed drinks and hard liquor was less common, with only a couple of women reporting this type of alcohol use (Figure 3.3, Table 1.3).

Number of alcohol units consumed during pregnancy was relatively low; most women (nine out of the ten who reported gestational alcohol use) only consumed 1 - 3

units of alcohol (bottles, glasses, or shots) during the entire pregnancy. The exception was one participant who reported an alcohol consumption rate of 1-3 alcohol units per month during the first trimester (Table 1.5).

Alcohol usage typically occurred during the first trimester, with the exception of four participants, who reported alcohol usage in the second or third trimester (Table 1.5).

Consumption of vitamins and medications:

Most women reported consuming prenatal vitamins during their pregnancies (83.4%). Eight point eight percent of women reported taking prescription morning sickness medicine, and 3.6% reported taking prescription pain medications. Acetaminophen was the highest used over-the-counter medication (38%). A small number of women reported taking aspirin (1.6%), ibuprofen (3.1%), decongestants (1.6%) and cough and cold medications (4.2%; Table 1.4) during their pregnancies.

Demographic factors and risky consumption habits:

Pearson correlation coefficients between demographic factors (age, education, and income) and dietary risk factors (consumption of tuna, salmon, canned foods, sugary desserts, fast foods, tap water, caffeine, and alcohol) were calculated. Women with lower income were more likely to have reported eating any canned foods during their pregnancies ($r = -0.143$; $p < 0.05$; $n = 191$). No other significant correlations were found.

Non-parametric Kruskal-Wallis Tests were performed to explore differences in consumption frequencies for fish, canned foods, sugary desserts, fast foods, caffeinated beverages, and alcohol with grouping variables of age, education, and income. Kruskal-

Wallis tests revealed no significant differences in consumption frequencies among groups.

DISCUSSION

Healthy eating and drinking:

Consumption of beef, chicken, tilapia, fruit, bottled or filtered water, milk, juice, and decaffeinated beverages were considered to be generally safe and healthy for the developing fetus. While recent risk factors associated with meat products have been described [344], the beneficial dietary supplementation of these choline-rich foods has been judged to outweigh the risks associated with hormone and antibiotic levels in certain meat and dairy products. Likewise, high-sugar content in certain types of juices may be associated with the onset of gestational diabetes; however, the vitamins and nutrients in juices merit their inclusion in the healthy drinking category. The American Dietetic Association recommends, for pregnant women, a healthy diet in accordance with the Dietary Guidelines for Americans (2005) including a variety of daily grains, milk products, fruits, vegetables, and iron-rich meats [293, 320]. The majority of pregnant women reported eating healthy foods including tilapia, beef, chicken, or pork, fresh fruit, milk, and juice during their pregnancies. The frequencies at which women in our sample ate these foods did not strictly adhere to daily recommendations. Only a third of women, for example, reported eating fruit 7 or more times a week.

Unhealthy consumption habits:

Consumption of tuna, salmon, canned goods, sugary desserts, fast foods, and drinking of tap water, caffeinated beverages, and alcoholic beverages during pregnancy have been deemed unhealthy due to the appearance of environmental toxins found to have harmful effects in the developing offspring.

Tuna:

The bioaccumulation of methylmercury (MeHg) in marine life, particularly tuna, presents a threat for developing fetuses whose mothers frequently eat this fish during their pregnancies, particularly because it is thought that mercury accumulates more readily in the fetal brain than in the maternal brain, interrupting patterns of cell fate, proliferation, migration, and neural outgrowth [181-183]. Because young children cannot metabolize mercury at the same rates as adults, exposure through either maternal or childhood consumption is of great concern. Even low levels of tuna consumption in children can readily result in blood mercury levels that exceed the health limit [345]. Epidemiological studies with cohorts from fish-eating populations have found that prenatal exposure to methylmercury has been associated with a myriad of developmental deficits involving attention, verbal learning, visuo-spatial and motor function, and delayed performance [191, 198]. The FDA reports that mean mercury levels of tuna range from 0.128 ppm (light canned tuna) to 0.689 ppm (big-eye tuna). However, mercury levels are highly variable and the FDA reported canned tuna to contain as much as 0.889 ppm for light canned tuna, and 1.816 ppm for big-eye tuna [209]. Currently, the FDA and the Environmental Protection Agency (EPA) recommend that pregnant women eat no more than six ounces of tuna per week. However, tests

performed by Consumer Reports on new samples of white tuna revealed that eating only 2.5 ounces of any of the new samples of white tuna would cause pregnant women to exceed the daily mercury levels that the EPA considers safe [346]. Worryingly, more than half of the women (52%) in this study reported consuming tuna during their pregnancies, suggesting that pregnant women are generally not aware of risk associated with tuna consumption.

Farmed salmon:

Polychlorinated biphenyls (PCBs) are lipophilic compounds found in fatty tissues of marine life feeding in contaminated waters. Staggering levels of PCBs have been found in farmed salmon compared to their wild-type counterparts, and contaminated commercial salmon feed has led to the bioaccumulation of these dangerous compounds [178]. Prenatal exposure of PCBs has been linked to lower birth weights, smaller head circumferences, and abnormal reflex abilities in newborns, as well as to mental impairment in older children [212, 214, 223, 347]. The FDA limits limiting PCB residues in fish to 2 ppm [348]. Although epidemiological studies seem to suggest PCB exposure is related to poor outcomes in neurodevelopment, the precise exposure patterns leading to these deficits have not been characterized [347]. However, a recent meta-analysis of 12 European studies found an association between fetal growth and PCB exposure at low, clinically-relevant levels [216].

More than a quarter of the women (25.5%) surveyed reported consuming salmon, a commonly ingested source of PCBs. Although prenatal fish intake, including tuna and salmon, can be a good source of Docosahexaenoic acid (DHA), a fatty acid thought to be beneficial in development, fetuses may be at risk for adverse outcomes,

and pregnant women should be advised to be selective about which fish they choose to consume, or seek supplementation with fish oil.

Canned foods:

Bisphenol A (BPA) found in the lining of metal cans used for food represents a danger to developing fetuses whose mothers consumed a diet high in canned foods. BPA has received recent attention as a controversial ingredient in child sippy cups, baby bottles, and reusable water bottles, leading to a ban by the FDA on the use of the plastic additive in sippy cups and baby bottles [179, 239]. Perhaps less well-known are the dangers of BPA exposure in consuming canned foods. Leaching of BPA from the epoxy resin of metallic food cans has been demonstrated in many studies [239, 270, 271]. Prenatal exposure to BPA has been found to be associated with higher externalizing scores (hyperactivity and aggression) in two-year-old females and reproductive effects in rodent models [255, 268]. The majority of women surveyed (73.9%) reported consumption of canned foods during their pregnancies, with 11.9% reporting consumption at least 4 times a week, suggesting women may not be aware of the dangers of BPA exposure from canned foods. Although epidemiological studies concerning prenatal BPA exposure are lacking, animal studies warn of the detrimental effects of BPAs. Children of pregnant women maintaining a diet high in canned foods are at risk for adverse postnatal outcomes. This study has found that low income is inversely correlated with canned food consumption, suggesting that women of low SES in particular may be especially at risk.

Sweet desserts:

Frequent consumption of sugary desserts during pregnancy may contribute to increased likelihood of gestational diabetes mellitus (GDM), a condition of glucose intolerance that has been implicated in many pregnancy problems including macrosomia, large for gestational age (LGA) infants, and increased rates of cesarean delivery [349-353]. Hispanic women in particular are two and half times more likely than non-Hispanic whites to suffer from GDM [354]. Increased sugar intake among pregnant adolescents has been linked to maternal gestational diabetes and LGA infants [353, 355, 356], and problems with gestational glucose control have been associated with neural tube defects [357]. On the other hand, carbohydrate restriction has been shown to aid in maternal glycemic control, alleviating some of the adverse pregnancy outcomes seen in patients with GDM [350]. More than a third of women (37%) ate sweet desserts more than one time per week, and 13.3% ate desserts more than 4 times per week. Children born of women maintaining a diet high in sweet desserts are at risk of macrosomia and postnatal obesity. Of course, women with GDM should be advised to closely monitor their sugar intake during pregnancy.

Fast foods:

The past decades have seen an insurgence of reliance upon high-energy, low nutrient foods that correlate with rising rates of obesity. A prenatal diet high in fast food represents a possible danger to the developing fetus, as these foods usually contain high levels of fat and salt. Maternal diets high in fat have been associated with increased likelihoods of postnatal diet-induced obesity in offspring, and have the potential to influence epigenetic markers leading to altered postnatal gene expression and eating

behavior [358, 359], Prenatal diets high in sodium levels have been linked to decreased gestational weight gain and an increased responsiveness to stress in adults [360, 361]. More than a quarter of women surveyed (25.7%) report eating fast foods at least once a week during their pregnancies. An alarmingly high percentage of surveyed women reported consuming fast foods more than four times per week (7.7%), and are at heightened risk for adverse fetal effects of high maternal salt and fat diets. Additionally, Hispanic populations are at risk for higher fat intakes from dairy foods [340]. Based on the literature, prenatal advising should stress the importance of eating a healthy diet low in these energy-dense, nutrient low foods so as to lower future generations' risk of obesity and stress conditions.

Tap water:

Drinking water has been found to have levels of many prenatal toxins including trihalomethanes (THMs), and certain drinking water disinfection by-products (dibromoacetic acids, or DBAs). The Drinking Water Quality Report for Downey, CA warns of 20 different pollutants in the city's water, with eight chemicals existing at concentrations that exceed the health guidelines set by federal and state agencies: tri- and tetro-chloroethylene (DBAs), alpha particle activity, arsenic, radium 228, lead, radium 226, and combined radium [362]. Many women in our sample (12.1%) reported drinking tap water during pregnancy, suggesting risk for exposure to many of these dangerous contaminants. Contaminants such as arsenic and DBA, and radium 226 have been found to result in central nervous system defects, oral cleft defects, and neural tube defects, and small for gestational age births, and risks for fetal death [278, 288, 363]. Women drinking tap water in areas where contamination is exceptionally high, as in

Downey, CA, are at risk for adverse outcomes resulting from prenatal exposure to certain chemicals. Pregnant women should instead be encouraged to drink filtered or bottled water.

Alcohol:

The spectrum of disabilities associated with prenatal alcohol exposure is termed FASD (Fetal Alcohol Spectrum Disorders). The prevalence of FASD has been difficult for researchers to ascertain. Some studies have found that anywhere from 0.5 - 2 cases per live births [80]. One study found as high as 1 per 100 live births when the full range of FASD was taken into account [364]. There is a large amount of data characterizing the severe complications in children who were exposed prenatally to alcohol. Children born with FASD often exhibit abnormal craniofacial features and have a litany of cognitive impairments including learning disabilities, decreased intelligence, decreased reaction time, slow sensory processing speed, language dysfunction, and behavioral disorders that are direct results of nervous system injury [121, 365-367].

Self-reporting of maternal drinking:

Information on the prevalence and pattern of maternal drinking has been notoriously difficult to ascertain. One possible diagnosis tool is the detection of biomarkers at birth. FAEEs (fatty acid ethyl esters) accumulate in the meconium after alcohol exposure in the first and second trimester [368]. Such methods have been useful in detecting alcohol usage in at-risk subjects at birth, but are impractical for use in a comprehensive assessment of average alcohol usage during the entire gestational period. Thus, although biological methods of measuring alcohol usage during pregnancy have

been developed, self-reports of maternal drinking remain the most effective methodology for maternal drinking prevalence assessments.

Ten women (5.8%) surveyed in Downey, CA reported drinking some time during their pregnancies. This number is slightly lower than national estimates of maternal drinking from the Centers for Disease Control and Prevention (CDC; 7.6% of pregnant women surveyed) [31]. Differences in rates may be due to the demographic make up of our sample. Maternal drinking rates are highest in white populations of older individuals (35-44 years of age) [31]. This study examined a population of relatively young (89.2% under 35) women who were predominantly Hispanic (87.4%). Methodological differences may also be at play. CDC data is obtained from the Behavioral Risk Factor Surveillance System (BRFSS), a telephone survey system that asks women to report alcohol usage within the last 30 days. Conversely, the current study surveys participants in late pregnancy or early post-pregnancy periods, when subjects might have difficulty remembering nutritional habits from early pregnancy. The chance of reporting inaccurate information from early pregnancy rises with the passing of time, and the stigma associated with maternal drinking might influence participants to deny usage if recall was suspect. However, despite the difficulties in obtaining accurate data through self-report, administration of a survey instrument to this population of women is the best way to obtain information regarding behaviors during pregnancy.

Methyl donors, prenatal vitamins, and alcohol consumption:

Research has consistently shown that intake of folate, choline, and other methyl donors are integral to the healthy development of the fetus. Specifically, the metabolism of these nutrients provides methyl groups in one-carbon methylation pathways [316].

Disruptions in one-carbon metabolism may result in decreased cognitive abilities [369] and serious birth defects [315]. Folic acid is an important contributor of methyl groups for pregnant women. A recent study has suggested that intake of prenatal vitamins may reduce the risk of autism [314]. Fortunately, intake of prenatal vitamins is fairly common: 83.4% of women report supplementing their diets with prenatal vitamins. Additionally, consumption of choline-rich foods was high: all women reported eating meat sometime during their pregnancies, and most reported eating meat at least once per week.

Because prenatal alcohol has been found to disrupt one-carbon metabolism, diets deficient in methyl donors may exacerbate the harmful effects of prenatal alcohol. Although women seem to be making an effort to curb their alcohol usage after recognizing their pregnancy, the number of women who report drinking during the first trimester is worrying (half of the ten women who reported drinking). Research has shown early pregnancy to be especially vulnerable to the teratogenic effects of alcohol, as deficiencies in methyl donor groups in the first month have been shown to result in neural tube birth defects in offspring [316].

Caffeine usage:

Caffeine is a xanthine alkaloid that can be found in coffee, sodas, energy drinks, and tea. Studies labeling caffeine as a teratogen date back to the late 1960s [370], and in 1980, the FDA advised limiting intake of caffeine during pregnancy, noting the substance's association with fetal mortality, birth defects, and decreased birth weights [371, 372]. The American Pregnancy Association recommends 150-300 mg as a safe daily dose of caffeine, although this is only based upon studies concerning risk of miscarriage [373]. Published epidemiological studies noting the impact of prenatal caffeine on child

behavioral and cognitive effects have been somewhat scarce and inconclusive [168, 374-376]. Animal studies, however, have found developmental delays, abnormal neuro-motor activity, and neurochemical disruptions, with some effects persisting until adulthood (for review, [377]). High levels of coffee consumption have been linked to fetal death after the second trimester [378, 379].

Caffeine has been consistently linked to abnormal motor activity and motor development [375, 376]. A majority of the subjects (80%) reported drinking caffeinated beverages during their pregnancies, with 14% of women reporting consumption of more than 4 caffeinated beverages per week. These numbers suggest that some children may be at risk for preventable persistent aberrations in neurochemistry and motor development.

Intake of over-the-counter and prescription medication

The number of women taking over-the-counter and prescription medications during pregnancy has increased within the past few decades [380]. A small number of women report using aspirin (1.6%) and ibuprofen (3.1%) during pregnancy. Animal experiments have implicated aspirin and ibuprofen, both cyclooxygenase inhibitors, in a number of adverse fetal effects including physical malformations [381, 382] and postnatal cognitive deficits [383].

A small number of women (3.6%) reported taking prescription pain medications during pregnancy. Fetal effects as a result of prenatal opioid exposure are poorly understood, but seem to be related to poor developmental outcomes [384]. Case control studies have made associations between prenatal use of opioid analgesics and congenital heart defects, the primary factor in birth-defect related infant mortalities [385].

Less than 10% (8.8%) of women reported using prescription anti-nausea medications. Use of anti-nausea medications have been found to be associated with acute non-lymphoblastic leukemia [386] although some FDA pregnancy category B drugs (considered safe in pregnancy) such as Zofran, are often prescribed for pregnancy related morning sickness. Our study did not differentiate between the pregnancy categories of anti-nausea medications used.

A paucity of conclusive research on the effects of prenatal exposure to prescription drugs may have led to the assumption that they are safe to prescribe. However, studies highlighting the possible dangers of prescription drugs, as well as some over-the-counter medications, suggest otherwise. Use of opioid analgesics and category C and above anti-nausea medications put pregnant women at risk for aversive offspring outcomes. In any case, women should be properly informed as to the possible dangers of prenatal exposure to these medications.

Changing consumption patterns after recognition of pregnancy:

Reports of patterns of consumption, that is, information about the amount of a substance consumed and the period during pregnancy in which it was consumed, can provide a window into commonplace beliefs about what habits are healthy during gestation. For example, most women who consumed alcohol during pregnancy reported doing so only in the first trimester. Given that a significant number of women (48.6%) confirm their pregnancies halfway through the trimester, it is likely that these women who report drinking during the first few months are doing so before realizing they are pregnant. In contrast, the majority of women consuming caffeine during pregnancy report continuing their consumption of these beverages well into their second and third

trimesters, suggesting that caffeine is not commonly regarded as harmful to the unborn fetus. The implications of this research are two fold: firstly, women of childbearing age hoping to conceive should be advised to eliminate all alcohol consumption, as effects of maternal drinking have dire consequences in the first trimester when the mother may not know she is pregnant. Additionally, every effort should be made during clinical prenatal care visits to inform pregnant women of the harmful effects of environmental toxins that can be readily transferred to the fetus as a result of uninformed, unhealthy consumption habits.

Study limitations:

Several problems exist in attempt to extract such sensitive data (such as gestational consumption of alcohol) from participants, with underreporting and recall bias as major hurdles to accurate data collection. The uncomfortable nature of questions concerning maternal drinking may lead to extensive underreporting of alcohol consumption. Underreporting is suspected to be a major issue in the field [295]. Nevertheless, self-reports of maternal drinking remain the most effective tool for sensitive assessments.

Unfortunately, our data was limited to women who agreed to participate in our survey, and we were unable to randomize samples. As survey distribution was offsite at a private medical group in Downey, CA, we do not have information on percentages of women who were not included in the survey. Small sample sizes for other ethnicities such as Whites, African Americans, and Asians made cross-ethnic studies unfeasible. Similarly, we were unable to conduct certain analyses on a low but significant population of women reporting alcohol use during pregnancy. The FBMIQ was designed

to be a short 5-minute study assessing percentages of women who may be at risk for certain unsafe consumption habits and as such it does not represent a complete dietary intake assessment. The scope of the survey limited us to frequency and trimester information only in major food categories, thus we were unable to report frequencies of subcategory items (prenatal vitamins versus prescription pain medication, for example). Additionally, although many substances in foods and beverages are thought to be teratogenic at high levels, specific dosages and patterns of exposure leading to adverse outcomes are not yet known. Developmental deficits as a result of prenatal exposure to environmental toxins at clinically-relevant levels, along with reviews and meta-analyses of the current literature, are important areas of future research that will help inform future prenatal guidance protocols.

CONCLUSIONS

The current study analyzes dietary habits of pregnant or recently pregnant women in Downey, CA, with particular emphasis on consumption intake of substances thought to be teratogenic in nature. Our main findings are summaries as percentages of women reporting consumption of unhealthy foods and beverages during pregnancies. For example, we found high numbers of Hispanic pregnant women consumed methyl mercury through tuna, PCBs through salmon consumption, BPA through canned goods, DBAs containing tap water, caffeine containing beverages, and alcohol containing beverages during pregnancy. We also found that large percentages of pregnant Hispanic women reported eating high sugar sweet desserts and high fat and salt fast foods more than once a week. A small number of women reported the use of certain non-

recommended over-the-counter medications such as aspirin and ibuprofen, as well as prescription medications with unsafe FDA pregnancy categories. These data reflect a remaining risk in certain populations for adverse outcomes in fetal development. Fortunately, a majority of the women surveyed report taking prenatal vitamins, which aid in the prevention of many neural tube defects. Additionally, percentages women reporting healthy consumption habits were generally high. In summary, our findings in a population of predominantly Hispanic women suggest high levels of consumption of substances that serve as potential teratogens to unborn children. Because we have not surveyed other populations of pregnant women, we do not know whether this is something unique to Hispanic women, or ubiquitous among women of multiple ethnicities. However, it is clear that prenatal medical professionals should discourage the consumption of dangerous foods, beverages, and medications that women commonly report consuming during pregnancy. In light of our consumption data here, prenatal professionals, including but not limited to OBGYNs, should be encouraged to instruct their pregnant patients about the dangers of hidden teratogens in our food supply, including but not limited to methyl mercury and PCBs in fish, caffeine and alcohol in beverages and BPA in canned goods.

CHAPTER THREE

Postnatal effects of prenatal nicotine exposure on body weight, brain size and cortical connectivity in mice

OVERVIEW

Maternal smoking results in myriad physical, cognitive, and behavioral effects in offspring due to prenatal exposure to nicotine. As the mammalian neocortex coordinates sensory integration and higher-order processes including cognition and behavioral regulation, it follows that cognitive and behavioral phenotypes of prenatal nicotine exposure (PNE) may correlate with, or stem from changes in anatomy and physiology of the neocortex. The current study uses a prenatal nicotine mouse model to determine effects of PNE on body weight, brain weight, brain length and development of neocortical circuitry, including thalamocortical afferents (TCAs) and intraneocortical connections (INCs). Although dam nutrition, dam weight gain and litter size were not significantly affected by nicotine treatment, PNE resulted in lower newborn birth weight, brain weight and length. Interestingly, the reduction of body weight, brain weight, and brain length observed in newborn PNE mice compared to control mice was no longer present at postnatal day (P) 10. A morphological study of somatosensory and visual TCAs and INCs shows no major defects in areal patterning of these connections. These data add to a growing body of literature

on the neurobiological effects of PNE by providing new information on the time course of PNE-related change in the postnatal brain.

BACKGROUND INFORMATION

Nicotine acts on nicotinic acetylcholine receptors (nAChRs) that are distributed throughout the brain and the nervous system. Subtypes of these receptors can be seen early in the gestational period and are differentially distributed across the course of development, suggesting a putative role of nAChRs in brain development [352, 387-390]. Prenatal nicotine exposure has been shown to disrupt neuronal maturation during development, even at a dose that does not affect maternal health or neonatal growth [77]. Nicotine is thought to have a generally inhibitory effect in the development of the central nervous system [41], perhaps through the modulation of neurotransmitter systems which have been shown to play a role in developmental growth by promoting or blocking neurite outgrowth [43, 391, 392]. Despite our understanding of mechanisms that underlie disruption of neural development, details of specific anatomical changes that result from prenatal nicotine exposure are not completely known.

The neocortex is organized into anatomically and functionally distinct areas that are intricately connected via intraneocortical connections, or INCs. These connections, along with thalamocortical afferents (TCAs), form a complex circuit that is critical for normal sensory processing, sensori-motor integration, and proper cognitive and behavioral development. Disruptions in INC formation may underlie phenotypic aspects of certain developmental disorders. For example, our laboratory has found that prenatal ethanol exposure leads to disruptions in INC patterning in a murine model of FASD [393]. Given the ubiquity of cognitive-behavioral deficits observed in children

who were exposed to nicotine during gestation, similar disruptions in the developing cortex might be present in mice prenatally exposed to nicotine. Specifically, we hypothesized that prenatal exposure to nicotine would generate an overall reduction in size of the developing brain and cause aberrant development of INCs, and possibly TCAs, resulting from exogenous activation of nAChRs during early development.

To test our hypotheses, a prenatal nicotine exposure (PNE) mouse model was created by injecting experimental timed-pregnant CD1 mouse dams with nicotine throughout the gestational period. Our laboratory has recently completed a thorough lifespan analysis of INC development in CD1 mice, and thus chose this strain for our model [394, 395]. In order to assess anatomical changes induced by PNE, we compared body weights, brain weights and brain lengths as well as patterning of thalamocortical (TCA) and intraneocortical (INC) sensory connections of control and PNE mice on the day of birth, termed postnatal day (P) 0. We compared body weight, brain weight, and brain length measures at additional time points throughout life (P10, 20, and 50) across groups to determine whether changes observed at birth persisted through adulthood. In all, our study contributes to a growing body of literature of the postnatal effects of prenatal nicotine exposure on brain and neocortical development.

MATERIALS AND METHODS

Mouse colony:

All breeding and experimental studies were conducted in strict accordance with protocol guidelines approved by the Institutional Animal Care and Use Committee

(IACUC) at the University of California, Riverside. Experimental and control mouse pups were bred from timed pregnant mice dams from a CD1 colony originally purchased from Charles River. All mice were housed in a standard cage, with 12 h–12 h light–dark cycle and given ad libitum access to standard chow and water. After pairing and confirmation of pregnancy (noon on the day of cervical plug visualization was set as gestational day (GD) 0.5), each male was removed from the cage. All timed-pregnant female mice were housed individually, weight-matched and divided into 2 groups, experimental (nicotine treated) and control. For staging of pups, day of birth was considered P0.

Dam nicotine administration:

Previous studies have described changes in offspring birth weight as a result of prenatal nicotine exposure, to free base nicotine, tobacco, and cigarette smoke, via dam treatment [17, 67, 396-399]. In our current study, by isolating the addictive component of nicotine from the wide spectrum of additives found in tobacco, we sought to examine some specific neurobiological effects of nicotine exposure on murine development.

Nicotine dosage and injection methods:

In previous experiments with rats, a dose of 2 mg/kg/day was used to mimic the blood plasma levels of nicotine in smokers [70, 400-402]. Specifically, previous data in rats indicate that a dose of 1.5 mg/kg/day results in plasma nicotine levels comparable to those of humans who smoke one pack of cigarettes per day. This dose has been found to be sufficient in producing neurochemical changes in the brain [63, 70].

We administered nicotine via injection, as oral administration of nicotine has been shown to be aversive [403, 404]. 99% free-base nicotine in a solution of 0.9%

physiologic saline was administered to experimental dams via subcutaneous (SC) injection at a volume of 10 μ L/g. These mice were injected twice daily with 2 mg/kg free base nicotine. In order to eliminate the confounding effect of stress induced by handling and injection, control dams were given sham SC injections of sterile 0.9% physiological saline twice daily, at a volume of 10 μ L/g. All morning injections were given from 9:00 to 10:00 AM and afternoon injections were given from 4:00 to 5:00 PM. Nicotine injection is one method of dam administration that has been used successfully by other researchers [24, 54, 397, 405, 406].

Dam measurement techniques:

Daily weight gain, food, and water intake:

Average daily maternal weight gain was calculated from weight measures recorded each morning throughout gestation. For each dam, an average of her daily maternal weight gain values from GD 0.5 to GD 17.5 was calculated. The mean of all average daily maternal weight gain values of injected-control dams was compared to the mean of all average daily maternal weight gain values of nicotine-treated dams by way of an independent samples t-test.

Food weights were recorded daily from GD 0.5 until birth. For each dam, an average of her daily food intake values from days GD 0.5 to 17.5 was calculated. The mean of all average daily food intake values of injected-control dams was compared to the mean of all average daily food intake values of nicotine-treated dams by way of an independent samples t-test.

On day GD 0.5, calibrated water bottles containing 400 mL of water were placed in cages of experimental and control dams. Daily water levels were assessed via

graduated marks on the bottles. For each dam, and average of her daily water intake values from days GD 0.5 to 17.5 was calculated. The mean of all average daily water intake values of injected-control dams was compared to the mean of all average daily water intake values of nicotine-treated dams by way of an independent samples t-test.

Litter size:

The number of pups in a litter for each experimental and control dam were counted and recorded on P0. Average litter size born to experimental and control dams was analyzed with an independent samples t-test.

Offspring tissue preparation:

P0 mice:

Newborn PNE (from nicotine-treated dams) and control (from injected-control dams) pups were first weighed, euthanized with pentobarbitol (100 mg/kg, intraperitoneal (IP)) and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (PFA), pH 7.4. All P0 brains were removed from the skulls, and separated from the spinal cord. Brain length measurements, from the tips of the olfactory bulbs to the posterior medulla, were taken using a micrometer. The brain was weighed and digitally imaged using a Zeiss Stereo Discovery V12 stereomicroscope with a digital high-resolution Zeiss Axio camera (HRm) using Axiovision software (Version 4.7) connected to a PC, for visual size comparison (Figure 4.4). After dorsal view imaging, brains were post-fixed for at least 24 h in 4% PFA and then hemi-sectioned for post-mortem tracing. Length and weight analyses were performed using ANOVA with factors of treatment and age, with post hoc planned contrasts.

P10, P20, and P50 mice:

A subset of newborn experimental and control offspring were allowed to survive until either P10, P20, or P50 when they were weighed, euthanized with a lethal dose of sodium pentobarbital (100 mg/kg, IP), and transcardially perfused with 4% PFA. All brains were removed from the skulls, measured, weighed, and digitally imaged as described above. Size comparisons were made by way of ANOVA with factors of age and treatment, as well as post hoc planned contrasts.

Anatomic tracing techniques:

Lipophilic dyes 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (DiI, Invitrogen) and 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide (DiA, Invitrogen) were used to characterize ipsilateral TCAs and INCs in single hemispheres of control and PNE newborn brains. DiI and DiA are carbocyanine dyes that produce high intensity labeling in post-mortem brains, and have a crystalline structure that allows for dye placements into developing sensory areas in neocortex, as has been shown previously [394, 395]. In each P0 mouse neocortical hemisphere, crystals of DiI and DiA were placed in two discrete locations: a rostral-parietal region of the cortex (putative somatosensory cortex) and a caudal-occipital region of the cortex (putative visual cortex). Methods for dye crystal placement have been described in detail elsewhere [394, 395, 407]. After dye placement, the tissue was placed into 4% PFA and stored in the dark at RT for approximately two to three weeks to allow for transport of tracers. Dye placement locations (DPLs) in sensory cortex of controls were confirmed via retrogradely labeled cells in the proper thalamic nuclei: putative somatosensory cortex DPLs were confirmed with label in the ventral posterior nucleus (VP) and putative

visual cortex DPLs were confirmed with label in the lateral geniculate nucleus (LGN). DPLs were matched across PNE and control hemispheres. After dye transport, hemispheres were embedded in a 5% low melting-point agarose in distilled water and sectioned on a vibratome in the coronal plane at 100 μ m. Sections were collected and counterstained with 4'-6-diamidino-2-phenylindole (DAPI, Roche Diagnostics), mounted on glass slides and coverslipped with Vectashield (Vector Laboratories, Inc.).

Documentation and analysis of cortical connections:

All P0 PNE and control sections were digitally imaged using a Zeiss AxioCam connected to a fluorescent compound Zeiss Axioscope and PC. Each section was imaged three times with a red filter for DiI, a green filter for DiA, and a blue filter for DAPI (excitation wavelengths: red: Cy 3–550 nm, green: GFP–470 nm, blue: DAPI–359 nm, emission wavelengths: red: Cy 3–570 nm, green: GFP–509 nm, blue: DAPI–461 nm). All 3 images were merged and saved in high-resolution format and select sections from representative cases were presented in a rostral-to-caudal series (see Figure 4.7). By comparing side-by-side images of dye tracing sections from both groups (control and PNE), shifts in INC position across the cortical map and developing sensory area borders can be easily observed and documented, aided by the illustration of 2D “flattened” lateral view reconstructions (Fig. 4.7: A6–C6). In these reconstructions, the dye placement location (DPL) and locations of DiI and DiA retrogradely labeled cell bodies, along with other anatomical landmarks, in each section were identified and plotted and morphed into a lateral view of the cortex. This method has been shown to be effective for INC positional analyses and the delineation of developing areal boundaries at very early murine ages [394, 395]. To further analyze whether projection patterns of

somatosensory and visual INCs were altered as a result of prenatal nicotine exposure, the spread of retrogradely labeled cells stemming from dye placements, termed 'projection zones' was measured along the rostral-caudal axis (Figures 4.8, 4.9). Rostral-caudal measurements of cortical cell spread ('S' and 'V' in Fig. 4.9) were expressed as a percentage of the overall cortical rostral-caudal length ('L' in Fig. 4.9). Projection zones of somatosensory and visual areas of PNE brains at P0 were compared to those of controls by way of independent samples t-test.

RESULTS

In this study, we created a prenatal nicotine exposure (PNE) mouse model in order to assess global changes in body weight and brain size from birth through adulthood, and whether a major anatomical feature of neocortical development, cortical circuitry, was altered in the newborn PNE mouse. We made systematic assessments of nicotine-treated dam nutrition, including measures of weight gain, food and water intake, and litter size. Despite no gestational nutritional differences between control and nicotine-treated pregnant dams, we observed significant reductions in body weight, brain weight, and brain length in PNE newborn mice. Interestingly, these effects were no longer present in P10 PNE mice. Additionally, there were no major shifts in nuclear position of retrogradely labeled TCAs from dye placements in somatosensory or visual cortex of P0 PNE mice. Analyses of INC position and related projection zones demonstrated no shifts due to prenatal nicotine exposure, despite the small brain size at birth. The reduced brain weight and length observed in P0 PNE mice does not persist. By P10, the brain size and length of PNE and control mice do not differ significantly.

This is a novel finding and may indicate the role of plasticity in the amelioration of cognitive deficits observed in humans exposed to nicotine in utero over time.

Dam nutrition

Daily weight gain, food and water intake of pregnant dams

Pregnant dams were weighed daily from GD 0.5 to 17.5 on a Fisher Scientific balance. Average daily weight gain was calculated in dams from injected-control ($1.1638 \text{ g} \pm 0.0640$) and nicotine treated ($1.2462 \text{ g} \pm 0.0738$) groups (Fig. 4.1). No significant differences were found between groups of dams (t-test, $p > 0.05$, $n = 6$ per group), suggesting that nicotine treatment does not alter gestational nutrition in dams as assessed by maternal weight gain.

Food intake measurements (in grams) were recorded daily from GD 0.5 to 17.5 in both groups, using a Fisher Scientific balance. No significant differences were found in daily food intake between injected-control ($6.32 \text{ g} \pm 0.22$, $n = 4$) and nicotine treated ($6.26 \text{ g} \pm 0.46$, $n = 6$) groups (Fig. 4.1; t-test, $p > 0.05$).

Average daily water intake (in mL) was calculated from water levels recorded daily in both groups from GD 0.5 to 17.5. No significant differences were found between injected-control ($6.24 \text{ mL} \pm 0.27$; $n = 4$), and nicotine treated ($9.48 \text{ mL} \pm 0.9$, $n = 6$) groups (Fig. 4.1; t-test, $p > 0.05$).

Litter size

Number of pups per litter for each dam was recorded on P0. No significant differences were found between litter sizes from injected-control ($11.2 \text{ pups} \pm 0.49$) and nicotine treated dams ($11.8 \text{ pups} \pm 0.73$, $n = 5$; t-test, $p > 0.05$; Fig. 4.1).

Offspring body weights

The body weights of control and PNE mice were recorded on P0, P10, P20, and P50 (Fig. 4.2). Average body weights at ages P0, P10, P20, P50 were $1.617 \text{ g} \pm 0.0241$ (n=12), $6.61 \text{ g} \pm 0.3053$ (n=10), $13.07 \text{ g} \pm 0.1202$ (n=6), and $33.89 \text{ g} \pm 0.9296$ (n=10), for control animals, and $1.44 \text{ g} \pm 0.0267$ (n=10), $6.44 \text{ g} \pm 0.1258$ (n=10), $12.56 \text{ g} \pm 0.5851$ (n=10), and $30.3 \text{ g} \pm 2.2938$ (n=8) for PNE animals, respectively (Fig. 4.2). An omnibus ANOVA with factors of treatment (control x prenatal nicotine) and age (P0 x P10 x P20 X P50) was conducted. Main effects of age regardless of treatment condition ($F(3,75) = 767.53$, $p < 0.001$) and treatment regardless of age ($F(1,75) = 10.34$, $p < 0.05$) were found, as well as a significant age by treatment interaction ($F(3,75) = 10.05$, $p < 0.05$). Post-hoc contrasts were conducted comparing body weights of control and nicotine animals at P0, P10, P20, and P50. As expected, P0 injected controls had significantly lower birth weights than the PNE group (t-test, $p < 0.05$, Fig. 4.2). However, this deficit was not observed past P0, as no significant differences in body weight were observed between control and PNE groups at P10, P20, or P50 (t-test, $p > 0.05$; Fig. 4.2), thus indicating that any impact of prenatal nicotine exposure on pup weight is transient.

Offspring brain weights

The weights in grams of control and PNE mice brains were recorded after sacrifice on P0, P10, P20, and P50. Control brain weights for P0, P10, P20, and P50 averaged $0.1096 \text{ g} \pm 0.002472$, $0.3546 \text{ g} \pm 0.01049$, $0.4750 \text{ g} \pm 0.00924$, and $0.48942 \text{ g} \pm 0.01014$ (n=10 per group), respectively. Mean weights of PNE brains for P0, P10, P20, and P50 were $0.0928 \text{ g} \pm 0.00274$, $0.3840 \text{ g} \pm 0.01279$, $0.4565 \text{ g} \pm 0.01216$, and $0.4891 \text{ g} \pm 0.0075$ (n=10 per group), respectively. An omnibus ANOVA with factors of treatment (control x

prenatal nicotine) and age (P0 x P10 x P20 x P50) revealed a main effect of age regardless of treatment ($F(3, 79)=745.23, p < 0.001$) but no main effect of treatment regardless of age. Calculations revealed a one-tailed significant interaction between age and treatment ($F(3, 79)=2.442, p=0.035$). Post-hoc contrasts were conducted comparing control and PNE brain weights across four ages. P0 control mean brain weight was significantly higher than PNE mean brain weight (t-test, $p < 0.01$, Fig. 4.3). However, no significant brain weight differences were found between control and PNE groups at P10 (t-test, $p > 0.05$), P20 (t-test, $p > 0.05$), or P50 (t-test, $p > 0.05$; Fig. 4.3).

Offspring brain size

Dorsal images of brains were taken after sacrifice, perfusion and dissection on P0 (Fig. 4.4: A1, A2), P10 (Fig. 4.4: B1, B2), P20 (Fig. 4.4: C1, C2), and P50 (Fig. 4.4: D1, D2). A marked reduction in overall brain size, and the size of the neocortex, between P0 control and PNE mouse brains was observed (compare Fig. 4.4, A1 and A2); however, this PNE-related reduction in brain size disappears by P10, where relatively no size difference between control and PNE brains at this age through adulthood at P50 can be observed (Fig 4.4: B1-B2, C1-C2, D1-D2).

Measures of brain length reveal reductions in PNE brain length at age P0, an effect that is no longer present by P10 (Fig. 4.5). Averages of brain length for control brains were $3.935 \text{ mm} \pm 0.06626$, $6.18 \text{ mm} \pm 0.06633$, $6.83 \text{ mm} \pm 0.05385$, $7.1 \text{ mm} \pm 0$ for ages P0, P10, P20, and P50, respectively ($n=10$ per group). Averages of brain length for nicotine exposed brains were $3.618 \text{ mm} \pm 0.08823$, $6.165 \text{ mm} \pm 0.06327$, $6.91 \text{ mm} \pm 0.06964$, and $7.16 \text{ mm} \pm 0.07483$ for ages P0, P10, P20, and P50, respectively ($n=10$ per group). An omnibus ANOVA with factors of age (P0 x P10) and treatment revealed a main effect of age regardless of treatment ($F(1, 39)=1082.41; p < 0.001$), a main effect of

treatment regardless of age ($F(1,39)=58.74$; $p<0.05$) and a significant interaction between age and treatment ($F(1,39)=4.294$; one-tailed $p<0.05$). Post-hoc planned comparisons revealed significant differences between brain lengths of control and PNE brains at P0 (Fig. 4.5; t-test, $p<0.05$). An omnibus ANOVA with factors of age (P10 x P20) and treatment revealed a main effect of age ($F(1,39)=121.101$; $p <0.001$) but no other effects, and an omnibus ANOVA with factors of age (P20 x P50) and treatment also revealed a main effect of age ($F(1,39)=18.435$; $p <0.001$) but no other effects. Brain length data are consistent with brain weights of offspring described in Fig. 4.3 and images shown in Fig. 4.4.

TCAs in control and PNE newborn mice

Our data document the PNE-related reduction in size of the whole brain and the neocortex on the day of birth, P0 (Figs. 4.3, 4.4). Although significant reductions in brain size can drastically impact function, the thalamocortical system also plays a critical role in normal brain development. Children prenatally exposed to high levels of nicotine via maternal smoking demonstrate abnormal development of high-level cognitive processing [408-410]. Disruptions in thalamic-cortical development could impact higher-level function in the developing nervous system and thus, if present, could potentially account for some aspects of human PNE. TCAs were analyzed to determine whether PNE disrupted the positional patterning of the TCAs in development. Placement of either DiI or DiA into somatosensory and visual cortical areas revealed no nuclear differences in thalamic retrograde labeling between control and PNE groups at P0 ($n=5$ per group). Thalamic nuclei were identified through a comparison of nuclear boundaries seen in the DAPI staining with a standardized mouse brain atlas [411]. Retrogradely

labeled cell bodies were observed in the ventral posterior (VP) nucleus of the thalamus in both control and PNE P0 brains (Fig 4.6: arrows in A1, A2) following dye placements into putative somatosensory cortex. Additionally, retrogradely labeled cell bodies were observed in the lateral geniculate nucleus (LGN) of the thalamus in both control and PNE P0 brains (Fig. 4.6: arrows in B1, B2) following dye placements into putative visual cortex. These data suggest that thalamic nuclear projections to specific dye placements locations within somatosensory and visual cortices are not dramatically disrupted in PNE mice. Specifically, the retrogradely labeled cells from visual cortex area DPLs in PNE and control brains are present in the same thalamic nucleus, the LGN. The same is true for retrogradely labeled cells from somatosensory cortex area DPLs in PNE and control brains, they are present in the same thalamic nucleus, the VP.

INCs of somatosensory and visual cortical areas at P0

The ipsilateral intraneocortical network is critical for proper development of the animal. This network integrates information from multiple sensory-receptor surfaces and seamlessly initiates sensori-motor function and, in newborn mice, defines developing area borders [394, 395]. A disruption of this network is hypothesized to underlie some behavioral developmental disorders [412-414]. To assess the impact of PNE on INC development, DiI or DiA crystal were placed in putative somatosensory and visual areas at P0 (Fig. 3.7: putative somatosensory: A2, B2, C2; putative visual: A5, B5, C5). After retrograde transport of tracers, labeled cell bodies in control and PNE groups were observed in sections counterstained for DAPI, presented in a rostral-caudal series in Fig. 3.7. In all control and PNE sections, retrogradely labeled cells resulting from DPLs into somatosensory cortex were located rostral and caudal to the

somatosensory DPL (Fig. 3.7: arrows, A1 and A3, B1 and B3, C1 and C3). In all control and PNE sections, retrogradely labeled cells resulting from DPLs into visual cortex were located rostral and medial to the visual DPL (Fig 3.7: arrows, A4, B4, C4). The raw data shown in the serial sections (Fig. 3.7: A3 and A4, B3 and B4, C3 and C4) show no overlap of the two dye colors. This demonstrates that projection zones of the discrete DPLs do not overlap in either the PNE or control mice. This is further illustrated in the 2d flattened reconstructions, or INC maps, which show the relative position of DPLs and INCs on a lateral view of the neocortex (Fig. 3.7: A6, B6, C6). Although the size of the cortex is reduced at P0 in the PNE mouse, the overall global patterning of somatosensory and visual INCs is not altered by PNE (compare: Fig. 3.7: A6 with B6, C6). Projection zones of somatosensory and visual cortices were measured in reconstructed INC maps of control and PNE brains at P0. Extent of rostral-caudal cell labeling from DPLs in somatosensory cortex averaged $45.96\% \pm 1.642$ of the cortical length for control animals, and $41.73\% \pm 1.279$ of the cortical length for PNE animals. Extent of rostral caudal cell labeling from DPLs in visual cortex averaged $44.43\% \pm 1.652$ of the cortical length for control animals, and $44.06\% \pm 2.434$ of the cortical length for PNE animals. Cell projection zone analyses in somatosensory and visual cortices, which represent a normalized analysis of spread of projection to a given DPL location, revealed no significant differences between control (n=5) and experimental (n=5) groups at P0 ($p < 0.05$; Figure 3.8: A, B). Although we replicate DPL size and location across cases, and verify location within a given sensory area using thalamic nuclear labeling, we cannot quantify dye uptake using this method of dye tracing in the post-mortem newborn brain. However, these methods of areal, lateral view reconstruction along with projection zone analyses can determine areal boundaries and the presence or absence of

major patterning defects in INCs due to PNE [394, 395]. Our data and analyses in multiple cases demonstrate no observed difference in somatosensory or visual INC patterning in the newborn PNE mouse neocortex.

DISCUSSION

In this study, we documented developmental effects in mice exposed to nicotine during the prenatal period. Dam nicotine treatment did not significantly affect daily gestational maternal weight gain, food intake, or water intake, nor did it alter litter size. Body weights, brain weights and brain lengths of PNE offspring were significantly reduced when compared to controls on the day of birth. However, these weight and size deficits were no longer present in P10 PNE mice; specifically, no body weight, brain weight or brain length differences were observed from P10-P50 between control and PNE groups. Analyses of retrograde labeling after dye placements in putative somatosensory and visual cortices at P0 revealed no PNE-related differences in thalamic nuclear input or patterns of intraneocortical connections.

Dam weight gain, nutrition and litter size is not affected by gestational nicotine treatment.

We did not observe nicotine-related changes in dam weight gain, food or water intake in our CD-1 mouse model, consistent with the findings of others [40, 70, 397]. By establishing that gestational dam nicotine treatment does not induce statistically significant differences in these measures, (see Fig. 3.1). Litter size is not typically affected by prenatal exposure to nicotine at any dosage [415-417], consistent with data presented in this report (Fig. 3.1).

Prenatal nicotine exposure results in reduced pup body and brain weight at birth, but normal body and brain weights are seen within 10 days of life in PNE mice.

Despite the wide variability in dosage, species type, and schedule of administration, repeated instances of low birth weights have been observed in prenatal nicotine research. Studies where rats were exposed prenatally to cycles of tobacco smoke have found lower birth weights in exposed animals versus controls [48, 418]. Similar results have been observed in rat models examining the effects of prenatal nicotine alone [67, 397]. In humans, low birth weights are common in newborns exposed to nicotine via maternal smoking throughout pregnancy [419] and [420] and lower birth weight is correlated with cognitive deficits [421]. It has been suggested that prenatal nicotine is activating the placental cholinergic system, which is in turn suppressing amino acid transport and diffusion of nutrients into placental circulation, leading to intrauterine growth retardation (IUGR) [47] and [53]. It may also occur because of vasoconstriction effects of nicotine during the prenatal period, which would return to normal after birth, as the significant reduction in body weight at birth is not observed at later ages in our data (P10, P20 and P50; Fig. 3.2).

Although a small number of studies report perinatal effects similar to what we have reported at P0 [40, 397]; see Figs 3.3, 3.5) our demonstration of progressive brain growth in the postnatal period following prenatal nicotine exposure is novel. Additionally, our results show that brains of P0 PNE mice were reduced in weight and length. Because reduced body weights were seen in P0 PNE mice as well, brain reductions could be correlated with growth retardation. Reduced numbers of cortical

cells in offspring prenatally exposed to nicotine have been previously reported [40]; thus, PNE related-reduced proliferation may contribute to lower brain weights and lengths in our PNE mice at P0. Acute administration of nicotine in neonatal rats has been shown to result in an inhibition of DNA synthesis, suggesting that nicotine is targeting and inhibiting pathways leading to mitosis, and promoting the premature cessation of proliferation and early onset of differentiation [422].

Pre and postnatal exposure to nicotine produces growth deficits in specific brain regions early in postnatal development [45, 423]. A comparison of brain weights and lengths of control and PNE brains at P10, P20, and P50 demonstrate no statistically significant differences, contrary to the significant reduction observed in PNE newborns (see Figs. 3.3, 3.4, 3.5). Analogously, overall brain size of PNE P10, P20, and P50 brains were comparable to controls. Recovery of brain size occurs early in postnatal life and correlates with a rapid increase in body weight, which also returns to control-levels by P10. These results mirror another study looking at postnatal brain weight in nicotine exposed rats, which found no differences in PNE brain weights at P40 [405]. However, this study is the first to comprehensively examine brain weights at multiple ages throughout postnatal development in a single PNE mouse model. There are some potential explanations for this recovery in the early postnatal period. Prenatal nicotine exposure results in lowered cholinergic activity postnatally, particularly in the second post-natal week [45], and increases in RNA and protein levels per cell were present in PNE cases, suggesting some level of cellular hypertrophy [40]. It appears that when early increased activation of NACHRs during the prenatal period returns to baseline postnatally, compensatory mechanisms may allow for increased cell proliferation and hypertrophy, which may, in turn, initiate the recovery of brain weight and size.

TCA and INC patterning are not altered by prenatal nicotine exposure.

The neocortex is comprised of functionally and anatomically distinct regions, the organization of which is integral to normal cortical function. Likewise, the pattern of interconnectivity within neocortex is extremely precise. Given the role of neocortex in governing higher-brain function, disruption of connectivity and regionalization during development may lead to prolonged cognitive and behavioral dysfunction. Indeed, analyses in a FASD mouse model document drastic disruption in intraneocortical connectivity [108]. Maternal smoking, and prenatal exposure to nicotine in particular, has been associated with behavioral, psychological, and cognitive deficits. It follows, then, that such dysfunction may correspond with alterations in the organization of the neocortex, such as those seen in the FASD mouse model.

This study examined TCAs and ipsilateral INCs in P0 brains of PNE mice, as compared to those born to dams sham-injected with physiological saline. Although we anticipated a shift in INC targeting as a result of PNE in our mouse model, there were no major differences in the retrograde labeling of TCAs in thalamic nuclei (VP or LGN, see Fig. 3.6) or INCs in the neocortex (see Fig. 3.7, 3.8). This is the first study to examine either TCA or INC development in a PNE animal model. Although no gross defects were discovered, single cell tracing or dendrite analysis may uncover abnormalities. Additionally, studies in older mice that can withstand in vivo tracing procedures may provide more detail on any potential defect in INC or areal patterning.

Exposure to nicotine versus tobacco smoke

Results suggest that nicotine alone is sufficient to alter birth body and brain weights, and brain size in mice prenatally exposed to nicotine. Despite the recovery of

brain and body weights in early postnatal development, the significant reductions we observed at birth implicate nicotine as a teratogenic substance. Often, pregnant women may quit smoking while pregnant, but continue to use nicotine replacement therapies (patch or gum) to avoid withdrawal. Elimination of carcinogenic substances present in tobacco smoke during pregnancy surely prevent unnecessary health risks in the fetus, however data presented here also strongly discourage the use of non-tobacco nicotine containing products.

CONCLUSIONS

Prenatal exposure to nicotine has teratogenic effects on mammalian offspring. In mice, prenatal nicotine exposure leads to decreased birth weight, brain weight and brain size. Although normal birth weights, brain weights and brain sizes are no longer present at 10 days of postnatal murine development, the long-term effects of the reduced birth body/brain weights and sizes are not known. Although we did not find any PNE-related alterations to TCA or INC patterning sensory areas in cortex, this is the very first study to document these connections in the PNE brain. Our data contributes to a growing body of literature on the deleterious effects of prenatal nicotine exposure.

CHAPTER FOUR

Prenatal nicotine exposure increases anxiety and modifies sensorimotor integration behaviors in adult female mice

OVERVIEW

Prenatal exposure to nicotine (PNE) has been associated with a myriad of physiological, cognitive, and behavioral effects in the developing offspring. In this study, CD-1 dams were given injections of nicotine or control vehicle throughout gestation and their offspring were raised to 6 months of age. Adult mice were administered a battery of behavioral tests (the Suok test, the elevated platform test, and the elevated plus maze test) to assess anxiety and sensorimotor integration. PNE resulted in a decreased likelihood of jumping during the elevated platform test and decreased directed exploration in the Suok test, both indicative of increased anxiety. Also, PNE mice showed increased numbers of missteps while traversing an elevated rod in the Suok test, demonstrating altered sensorimotor integration. No significant differences were found in falls, segments traveled, latency to leave the central zone, vegetative responses, risk assessment behaviors, or autogroom behaviors. The elevated plus maze test revealed no significant differences between groups. No significant differences in body and brain weights, or cortical thickness within motor, somatosensory, and visual

cortices were observed between PNE and control mice. Although neuroanatomical effects of PNE may be rescued as development progresses, defects in sensorimotor integration and increased anxiety persist into adulthood.

BACKGROUND INFORMATION

The developing brain is far more susceptible than the adult brain to the deleterious effects of toxins [424, 425]. Nicotine activates and desensitizes nicotinic acetylcholine receptors (nAChRs) throughout the brain and is thought to exert its effects by targeting cholinergic systems during brain development [44, 415]. We have reported previously that PNE impacts brain size at birth, resulting in a reduced cortical length [52]. Because the neocortex is the part of the brain responsible for higher cognitive function, we hypothesized that PNE induced alterations in cortical development may underlie the behavioral phenotypes observed in children exposed to nicotine during the prenatal period. Based on this and our previous research, we hypothesized that prenatal nicotine exposure (PNE) impacts normal development of the sensory and motor cortices, leading to abnormal behavior and increased anxiety. We further predicted that subtle changes in neocortical thickness and anatomy would be detectable in adulthood and that these abnormalities would be correlated with deficits in sensorimotor behavior and increased anxiety.

The current study used a CD-1 mouse model to investigate the long-term anatomical and behavioral effects of prenatal nicotine exposure (PNE) in 6-month old female mice. Control and experimental animals were subjected to behavioral assays of sensory and motor function as well as anxiety. Body weights, brain weights, and cortical

sizes were recorded, and thicknesses of sensory and motor cortices were measured. Although much is known about the impact of PNE on early development and adolescent outcomes, little research has examined consequences of maternal smoking and PNE that persist well into adulthood. This study confirms the behavioral teratogenicity of nicotine in a mouse model.

MATERIALS AND METHODS

To test our hypotheses, profiles of anxiety and sensorimotor integration in adult PNE and control mice were assessed through the use of behavioral assays, namely, the Suok test, the elevated plus maze test, and the elevated platform test which gauge the animals' anxiety levels as well as their ability to integrate sensory inputs with motor outputs in order to maintain balance [426-428]. After behavioral testing, animals were weighed and sacrificed, and their brains were removed. Neuroanatomical measures of brain weight, cortical width and length, and cortical thickness of motor, somatosensory, and visual cortices were measured.

Mouse colony

All breeding and experimental studies were conducted in strict accordance with protocol guidelines approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, Riverside. Experimental and control mouse pups were bred from timed pregnant mice dams from a CD1 colony originally purchased from Charles River. Mice were housed in a standard cage, with 12h-12h light-dark cycles and given ad libitum access to water and standard chow. After pairing and confirmation of pregnancy (noon on the day of cervical plug visualization was set as

gestational day (GD) 0.5), each male was removed from the cage. All timed-pregnant female mice were housed individually and divided into experimental (nicotine treated) and control groups. For staging of pups, day of birth was considered P0. Control and experimental female pups were weaned at P21 and housed with same-sex litter mates until they reached six months of age, at which point they underwent behavioral tests and sacrifice.

Dosage and nicotine administration

Doses of 1.5- 2 mg/kg/day of nicotine in rats are sufficient in producing neurochemical changes in the brain and have been shown to result in plasma nicotine levels similar to humans who smoke one pack of cigarettes a day [63, 400, 403]. Given the difference in human versus rodent pharmacodynamics, higher dosages in rodent models may be needed to mimic effects in humans [429]. Thus, previous studies have typically used dosages of 2-6 mg/kg/day to mimic the blood plasma levels of nicotine in moderate and heavy smokers [70, 401, 402, 415, 430].

Nicotine was administered via injection due to concerns about the palatability of nicotine, which has been shown to be aversive to rodents [403, 404]. 99% Free-base nicotine in a solution of 0.9% physiologic saline was administered to experimental dams via subcutaneous (SC) injection at a volume of 10 μ L/g. These mice were injected twice daily with 2 mg/kg free base nicotine, yielding a total dose of 4 mg/kg/day administered throughout gestation from GD 0.5 until P0. Control dams were given sham SC injections of sterile 0.9% physiological saline twice daily, at a volume of 10 μ L/g, to control for stress induced by handling and injection. Morning injections were given from 9:00-10:00AM and afternoon injections were given from 4:00-5:00PM. This schedule of

administration is in accordance with a CD-1 PNE model used previously in our laboratory [52].

Behavioral tests

Behavioral tests in animal models are an invaluable tool to the diagnosis, understanding, and treatment of developmental deficits resulting from prenatal exposure to nicotine. At six months of age, animals underwent behavioral testing to investigate the impact of prenatal nicotine on sensory and motor function and anxiety. A sample size of 8-10 animals per group from four litters was used. All behavioral testing took place between 10:00AM – 1:00PM. After acclimating to the dimly lit behavioral testing room for one hour, each mouse in turn was subjected first to the Suok test, then to the elevated plus maze, and the lastly to the elevated platform test.

The Suok test

The Souk apparatus was constructed in accordance with the specifications published in Nature Protocols [428] and consisted of an aluminum rod, 2-meters in length by 2-inches in diameter, suspended between two vertical squares of plexiglass (50 x 50 cm²). The rod is separated into two lengths of several 10-cm long segments on either side of a 20-cm long central zone (all marked with colored tape on the underside of the rod). The novel Suok test measures exploration, risk assessment behaviors, and sensorimotor integration; it was developed to target behavioral abnormalities that arise from pathways mediating anxiety and vestibular function. Behaviors are scored as the animal walks along a 2 meter-long rod for 5 minutes. Horizontal beams have previously been used in behavioral tests profiling balance and vestibular proficiency [431-433]. Similarly, the Souk makes use of a slippery, aluminum rod to pinpoint deficits in

sensorimotor integration. Concurrent measurements of reduced exploration are indicative of increased anxiety [434-436]. The Suok test is a novel test that targets stress or anxiety-evoked alterations in sensorimotor integration. Given that individuals with PNE are characterized by depression/anxiety disorders [437], the Suok presents itself as an appropriate behavioral assay to relate animal behavior correlates to human phenotypes.

At the start of each 5-minute testing period, animals were placed on the central zone with their snouts facing either end of the rod. Mice that fell off the rod were quickly repositioned on the apparatus in the same position and location. Several measures of behavior were observed and scored: (1) sensorimotor ability, as measured by number of missteps and falls from the rod, (2) locomotor activity, as assessed by number of segments travelled, (3) exploration activity, which includes directed exploration, latency to leave the central zone, risk assessment behaviors (full-body sniffing stretches; RABs), (4) vegetative responses (number of urinations and defecations), and (5) autogrooming behaviors. Differences between the two groups were analyzed with the use of independent samples t-tests.

The elevated plus maze test

The elevated plus maze has been validated as a useful technique for assessing anxiety related behaviors, relying on the animals' unconditioned fear of heights and inclination towards dark enclosed spaces [434]. Extended open arm activity reflects anxiolytic behaviors, whereas an increase in closed arm activity marks anxiety-related behaviors. The plus maze apparatus for this study was constructed in accordance with the dimensions specified in Nature Protocols [436]. The plywood plus maze consists of

four arms in a cross configuration (two open and two closed arms enclosed by 15-cm-high walls), each 30 cm long and 54 cm wide. The apparatus is elevated 50 cm from the floor.

Approximately 30 minutes after undergoing testing on the Suok rod, each animal was placed into the center of the elevated plus maze, facing an open arm. Time spent in open arms, closed arms, and center were recorded and analyzed by way of independent samples t-tests.

The elevated platform test

The use of elevated platforms has been used previously as a psychological stressor without using painful stimuli [434, 438]. The elevated platform apparatus consists of a vertical metal pole, 2 feet high and 2 inches in diameter, with a styrofoam stage at the bottom to cushion the fall of mice. This test can be used to test exploratory behavior as a function of the number of seconds the animal remains on the top of a small diameter vertical pole without jumping or climbing down.

Approximately 30 minutes after testing on the elevated plus maze, 6-month-old control and experimental mice were placed upon the vertical pole. The number of seconds (up to 60) the animal remained atop the pole was recorded, and differences between groups were analyzed using independent samples t-tests.

Neuroanatomical measures

Offspring body and brain measurements and tissue preparation

After behavioral testing, 6-month-old control and experimental animals were weighed, euthanized with a lethal dose of sodium pentobarbitol (100 mg/kg, intraperitoneal injection), and transcardially perfused with 4% paraformaldehyde in

0.1M phosphate buffer (PFA). Brains were removed from skulls, weighed, and whole brains were imaged using a digital high-resolution Zeiss Axio camera (HRm) using Axiovision software (Version 4.7) connected Zeiss Stereo Discovery V12 stereomicroscope and a PC. Cortical widths of brain hemispheres were measured along an axis perpendicular to the longitudinal fissure, extending to the most lateral edge of the cortex. Cortical lengths of hemispheres were measured from the most rostral tip of the cortex to the most caudal part of the cortex in parallel with the longitudinal fissure. Both cortical widths and cortical lengths were determined with the use of a micrometer. Brains were cryoprotected in a 30% sucrose/PFA solution for 3 days, after which they were sectioned on a cryostat on a coronal plane at 40 μm . Mounted sections were then dried and stained with cresyl violet (nissl) for assessment of anatomical features.

Measurements of cortical thickness

Identification of putative motor, somatosensory and visual cortices in nissl stained sections was conducted with the use of the second edition of Franklin and Paxinos' The Mouse Brain in Stereotaxic Coordinates [411]. Sections analyzed for sensory or motor cortical thickness were standardized at specific levels along the rostro-caudal axis (indicated by distance from Bregma according to Paxinos and Franklin, 2004). Motor cortex was identified at the rostro-caudal level immediately rostral to the genu of the corpus callosum (approx 1.18 mm from Bregma). Cortical thickness was measured along a line perpendicular to the tangent of the pial surface extending to the transition between layer VI and the tip of the cingulum (see Fig. 4.3: A1 and B1). Cortical thickness of somatosensory cortex was measured with a line perpendicular to the tangent of the pial surface extending through the visibly nissl stained barrel fields of the

somatosensory cortex lateral to the lateral ventricle and at a rostro-caudal level approximately -1.58 mm from Bregma (see Fig. 4.3: A2 and B2). Measurements for cortical thickness of visual cortex were taken along a line perpendicular to the tangent of pial surface extending to the transition between layer VI and white matter just lateral of the lateral thinning of the dorsal hippocampal commissure at a rostro-caudal level approximately -2.80 mm from Bregma (see Fig. 4.3: A3 and B3).

RESULTS

In this study, we investigated the long-term behavioral and anatomical effects of prenatal exposure to nicotine using a CD-1 mouse model. At six months of age, control and PNE mice underwent the Suok test, the elevated platform test, and the elevated plus maze test to assess sensorimotor function and anxiety. PNE mice were found to have an increased number of missteps and lowered incidences of directed exploration in the Suok test and jumping in the elevated platform test. These results revealed abnormal sensorimotor integration and increased anxiety in PNE mice. Mice were weighed prior to sacrifice and brains were extracted and analyzed for anatomical differences. Anatomical measurements of body weight, brain weight, cortical length and width, and cortical thickness reveal no significant differences as a result of prenatal exposure to nicotine.

Behavioral measures

Behavioral measures of sensory and motor function

Control and PNE mice at six months of age underwent behavioral testing on the Suok test. The number of missteps, falls, and the number of segments traveled while on

the Suok rod, were recorded (Fig. 4.1). The number of missteps, or the number of times a foot slipped while six-month old mice were traversing a metal rod during the 5 minute Suok test, were recorded. Mice prenatally exposed to nicotine displayed an increased number of missteps while traversing the rod (19.5 ± 1.43 missteps; $n=8$) compared to controls (7.6 ± 2.77 missteps; $n=10$; t-test; $p=0.00205$; Fig. 4.1: A). No significant differences in number of falls were detected between control (3.4 ± 0.73 falls; $n=10$) and PNE mice (2.5 ± 0.63 falls; $n=8$; t-test, $p=0.335$; Fig. 4.1: B). Control and PNE mice were also similarly active; control mice averaged 136.9 ± 16.76 segments travelled ($n=10$), and PNE mice averaged 164.5 ± 18.75 segments travelled ($n=8$; t-test, $p=0.290$; Fig. 4.1: C).

Behavioral measures of anxiety

Control and PNE mice underwent behavioral testing at 6 months of age to assess anxiety. For the elevated platform test, the number of seconds mice remained atop a vertical pole without jumping was recorded. For the Suok test, the following measures of anxiety were recorded: directed exploration, latency to leave the central zone, number of defecations/urinations, incidences of risk assessment behaviors (RABs), and incidences of autogrooming behaviors. For the elevated plus maze, the amount of time spent in the open and closed arms of the maze was recorded (Fig. 4.2).

All PNE mice failed to move from the elevated platform for the duration of the test (time on platform for each mouse totaled 60 seconds for $n=8$), whereas control mice remained atop the platform for a significantly shorter period of time (25.3 ± 6.27 seconds; $n = 10$; t-test, $p=0.000371$; Fig. 4.2: A). PNE mice also displayed significantly less directed exploration than control mice (81.88 ± 6.66 head movements versus 99 ± 3.47 head movements for PNE and control mice, respectively; $n=8$, $n=10$; t-test, $p=0.0441$; Fig. 4.2:

B). Average latencies to leave the central zone were 20.23 ± 4.81 seconds for control mice (n=10) and 36.0 ± 12.08 seconds for PNE mice (n=8) and were not significantly different (n=10 and n=8, respectively; t-test, $p=0.255$; Fig. 4.2: C). Groups did not significantly differ in number of urinations and defecations (1.4 ± 0.45 defecations for control and 1.25 ± 0.37 defecations for PNE; n=10 and n=8, respectively; t-test, $p=0.692$; Fig. 4.2: D). Incidences of RABs for the groups were similar. Control mice averaged 1.2 ± 0.25 RABs and PNE mice averaged 2.25 ± 0.70 RABs (n=10 and n=8, respectively; t-test, $p=0.193$; Fig. 4.2: E). The two groups were not significantly different in number of autogroom instances (Control, 0.4 ± 0.22 instances, n =10; PNE, 0.625 ± 0.26 instances, n=8; t-test, $p=0.525$; Fig. 4.2: F). The time spent in open and closed arms was recorded during a 5-minute elevated plus maze. The groups did not significantly differ in time spent in open (Control, 7.8 ± 3.49 s; PNE, 9.82 ± 3.61 seconds) or closed (Control, 236.4 ± 14.03 s; PNE, 250.7 ± 10 seconds) arms (n=11; t-test, $p=0.789$ and $p=0.153$ for open and closed arms, respectively; Fig. 4.2: G and H).

Assessment of possible litter effects

To assess whether litter effects exist in our sample, we conducted analyses of all behavioral measures using litter as the unit of analysis, rather than single offspring as individuals. Significant differences were found for missteps ($p=0.0498$, n=4 for control and n=4 for PNE), directed exploration ($p=0.0264$, n=4 for control and n=4 for PNE), and elevated platform ($p=0.00621$, n=4 for control and n=4 for PNE), confirming our original findings that are presented in the figures using the individual offspring as the unit of analysis. No significant differences were found for falls ($p=0.110$, n=4 for control and n=4 for PNE), segments travelled ($p=0.165$, n=4 for control and n=4 for PNE), latency to

leave central zone ($p=0.164$, $n=4$ for control and $n=4$ for PNE), defecations and urinations ($p=0.466$, $n=4$ for control and $n=4$ for PNE), RABs ($p=0.164$, $n=4$ for control and $n=4$ for PNE), autogroom ($p=0.401$, $n=4$ for control and $n=4$ for PNE), time in open arms ($p=0.353$, $n=4$ for control and $n=5$ for PNE), and time in closed arms ($p=0.0169$, $n=4$ for control and $n=5$ for PNE). These findings using litter as the unit of analysis are also consistent with our original analyses.

Physiological and neuroanatomical measures

Brain and body weights

The body weights of control and PNE mice were recorded at 6 months of age (Fig. 4.3: A). Average body weights for control mice were 35.5 ± 1.16 g ($n=10$) and average body weights for PNE mice were 33.125 ± 0.93 g ($n=8$). No significant differences in body weights were found between control and PNE mice (t-test; $p=0.129$).

After behavioral testing, animals were sacrificed, their brains extracted and weighed. Average brain weights for were 0.5278 ± 0.0106 g for control mice ($n=6$), and 0.5430 ± 0.0146 g for PNE mice ($n=8$; Fig. 4.3: B). No significant differences in brain weights were found between control and PNE mice (t-test, $p=0.422$).

Cortical measurements of width and length

Dorsal images were taken of 6-month-old control and PNE brains after sacrifice and perfusion (Fig. 4.4: A and B) and appear to be similar in cortical size. Control brains had an average cortical hemisphere width of 5.4 ± 0.117 mm ($n=5$), while PNE brains averaged 5.475 ± 0.0701 mm ($n=8$; Fig. 4.4: C). Cortical length measurements averaged 9.2 ± 0.145 mm ($n=5$) for control brains and 9.09 ± 0.0611 for PNE brains ($n=8$; Fig. 4.4:

D). Measurements of cortical size reveal no differences in cortical width or length (t-test, $p=0.915$ and $p=0.601$ for width and length, respectively).

Measures of cortical thickness in sensory and motor areas

Six-month-old control and PNE brains were sectioned on a cryostat at 40μ and stained with cresyl violet to assess anatomical differences. Representative nissl-stained sections are shown for analysis of motor (Fig. 4.5: A1 - control, and B1 - PNE), somatosensory (Fig. 4.5: A2 - control, and B2 - PNE), and visual (Fig. 5: A3 - control, B3 - PNE) cortices. Double-headed arrows signify location of cortical thickness measurements. Cortical thickness measurements revealed no significant differences in cortical thickness of motor cortex (1.236 ± 0.0411 mm and 1.268 mm for control and PNE, respectively; $n=3$; t-test, $p=0.604$; Fig. 4.5: C1). Cortical thickness for somatosensory cortex was measured in control and PNE sections. Control mice with an average thickness of 1.077 ± 0.025 mm did not significantly differ from PNE mice with an average thickness of 1.158 ± 0.029 mm ($n=3$; t-test, $p=0.104$; Fig. 4.5: C2). Cortical thickness measures for visual cortex were 0.7 ± 0.025 mm for control brains and 0.774 ± 0.029 mm for PNE brains ($n = 3$; Fig. 4.5: C3). No significant differences in visual cortex thickness were detected (t-test, $p=0.158$).

DISCUSSION

This is the first study to document long-term alterations in an adult 6-month old mouse model of prenatal nicotine exposure. We sought to evaluate behavioral differences in sensory and motor function and anxiety and correlate these observations with anatomical differences. Prenatal nicotine has been found to effect measures of both

sensorimotor integration and anxiety. This study suggests that despite the apparent recovery in brain and body weight in PNE animals (found to be decreased at birth, see [52], gestational nicotine exposure has long-term behavioral effects that persist well into adulthood. While this study found no gross anatomical changes in the brain, others have found more subtle differences in the cortex that may relate to the behavioral changes seen in this study. For example, PNE has been shown to result in changes in dendritic branching, dendritic length, and spine density in the cortices of exposed female rats at age P100 [439]. PNE has also been found to alter expression of nAChR subunits in P75 rats exposed to nicotine [402].

Sex, age, and animal model considerations of prenatal nicotine exposure

Previous research in PNE offspring has suggested that males and females display different patterns of effects from prenatal nicotine exposure. Sex differences in behavior, sensory function, catecholamine systems, psychopathology, and cortical thickness have been found as a result of such exposure [64, 416, 440, 441]. The current study evaluates behavioral and anatomical changes in female mice only, so as to control for differential sex effects. It is important to note that the results of this study may only be generalized to females, and further research is needed to investigate the long-term anxiogenic and sensorimotor function effects of PNE in males.

Most research has been conducted on the behavioral and physiological effects in early postnatal development, adolescence and early adulthood [14]. With the exception of a few studies [402, 437], little research has examined the long-term effects of prenatal nicotine in mice later in adulthood (past two months of age). This study sought to

characterize the neuroanatomical and behavioral changes that persist past early adulthood and into later adulthood.

The PNE model in this study uses an injection method for administration of nicotine. Some researchers have found the injection method to be clinically aversive, leading to decreases in gestational body weight gain and food intake [442]. However, previous work in our laboratory with this PNE model found no changes in food intake or weight gain [52]. Acute doses of nicotine may induce sudden increases in blood nicotine concentration, leading to hypoxia and ischemia. Changes in locomotor activity resulting from the injection method have been reported, but results are conflicting [399, 442]. This study found no significant differences in locomotor activity between experimental and control animals.

Long-term disruption in sensory and motor function is observed in 6-month-old mice prenatally exposed to nicotine

Studies in humans and rodents have revealed impairments in sensory and motor processing as a result of prenatal nicotine exposure. Reduced prepulse inhibition responses were found in female, but not male, rats exposed to prenatal nicotine [440], and deficits in sensorimotor reflexes were found in neonatal nicotine exposed mice [24]. Numerous impairments in auditory processing have been observed in children and adolescents prenatally exposed to tobacco [443-445] and may be related to sensory processing deficits as opposed to difficulties in sound detection [446, 447]. Fine motor skills are disrupted in neonates exposed to maternal tobacco [21, 23]. These studies have assessed sensory and motor function early in development and into adolescence. Little is known, however, about sensory and motor function in later adulthood with prenatal

nicotine exposure. Our results indicate that PNE-induced disruption of sensorimotor integration can be seen well into adulthood. While the number of falls for control and experimental 6-month-old were not affected by gestational nicotine, PNE mice displayed a significantly increased number of missteps while traversing the Suok rod, suggesting an inability to integrate sensory information with motor control. Prenatal nicotine has been shown to disrupt cholinergic activity in the developing brain and may be inducing sensorimotor deficits via altered cholinergic modulation of spontaneous activity in the development of sensory systems [45, 448, 449].

Previous research has documented a link between prenatal nicotine in rodents and postnatal hyperactivity in adolescence and early adulthood [24, 450-452]. Similarly, children with mothers who smoked during pregnancy exhibit higher incidences of ADHD [453]. Studies in older mice (up to P100) have also found heightened locomotor activity [454]. In contrast, analyses of segments traveled during the Suok test reveal no differences in activity in 6-month old mice, suggesting a return to baseline locomotor activity as offspring progress through adulthood. These results could also be explained by our exclusion of male PNE mice, as differential effects of prenatal nicotine on the hyperactivity of young males and females have been previously documented [64].

The Suok is specifically designed to investigate the interplay between sensorimotor integration and anxiety. Indeed, clinical studies and research on neural circuitry and monoaminergic systems have found processes concerning vestibular function and emotional regulation to be related [455, 456]. This study finds that PNE results in deficits in both sensory and motor function as well as in anxiety.

The extent of mediation exerted by PNE on this vestibular-anxiety system merits further research. Given the Suok test's specific targeting of the interplay between anxiety

and sensorimotor integration, deficits in sensorimotor function may be especially apparent during times of duress.

Long-term effects of prenatal nicotine on behavioral measures of anxiety are observed in 6-month old mice

Prenatal nicotine exposure has been correlated with the emergence of neuropsychiatric disorders in adolescence [14, 27], perhaps due to the developmental effects of introducing increased levels of nicotine into the catecholaminergic systems. Studies investigating anxiety-related behaviors in animal models have revealed conflicting results. Ajarem and Ahmad found an anxiolytic effect of PNE in P35 mice subjected to the elevated plus maze [24], while Eppolito et al. (2010) found no anxiety-related effects in P32 mice. However, anxiety and depressive-like symptoms have been documented in P72 adult [402] and aging 12-14 month old rats [437] prenatally exposed to nicotine.

The current study examines anxiety-related behaviors in 6-month-old female mice prenatally exposed to nicotine. These mice were found to exhibit decreased instances of directed exploration in the Suok test, and failed to move from the elevated platform within the allotted time frame in the elevated platform test, suggesting that gestational nicotine may have a late-term anxiogenic effect.

Several hypotheses may account for such results. First, PNE-induced anxiety may be age-dependent, as adulthood may render PNE offspring more susceptible to stress-related decreases in exploratory behaviors. Difficulties in detecting anxiety-related behaviors shortly following sexual maturation may also arise as a result of the hormonal and physiological changes accompanying adolescence, accounting for the lack of

anxiety-related behaviors in young adult mice documented in previous research [24, 402]. Sex differences in anxiety-related behaviors have also been reported, which may play a role in the detection of such behaviors at certain developmental time points [457]. Our results therefore specifically reflect the long-term effects of prenatal nicotine on females. Differences in maternal care as a result of prenatal nicotine exposure may also play a role, as cross-fostering of PNE offspring has been shown to have an impact on anxiety-related behaviors later in life [452].

Surprisingly, the elevated plus maze test revealed no effect of PNE on anxiety, while decreased measures of reduced directed exploration in the Suok Test and jumping or climbing behaviors in the elevated platform test suggest otherwise. This could be explained by differences in anxiety detection between the two kinds of tests. Previous studies suggest that open space, rather than novelty or height, is the primary anxiogenic factor in the elevated plus maze [458, 459]. Perhaps the Suok and the elevated plus maze tests are more sensitive to subtle behavioral manifestations of anxiety because they reflect novelty or height-induced stressors. Additionally, the Suok and the elevated platform test detect measures of stress-induced anxiety because the animal is forced to respond in open, elevated environments, as opposed to the elevated plus maze, wherein animals may choose to spend time in dark, enclosed arms. Our findings suggest that prenatal nicotine targets long-term disruptions in anxiety-related behavior in stressful environments.

Carryover effects in behavioral testing

In this study, animals underwent the Suok test first, the elevated plus maze second, and the elevated platform test third. The sequence of behavioral testing may

have affected observations of behavioral change in the elevated plus maze and elevated platform tests. Carryover effects resulting from prior behavioral experience have been shown to impact subsequent testing [459, 460]. Although previous exposure to various open arm/closed arm configurations has been shown to result in heightened avoidance of open arms in subsequent testing, previous experience with open arm configurations (which most closely resembles the Suok) produced no apparent changes in behavior, presumably because no avoidance learning is involved with such exposure [459]. By subjecting mice to the Suok before the elevated plus maze test, we hoped to alleviate any possible carryover effects in the elevated plus maze. The extent to which previous experience with the elevated plus maze modified responses in the elevated platform test, however, remains unclear. Previous training with the elevated plus maze has been found to impact exploration in subsequent plus maze testing, but had no impact on open-field exploratory behavior [459, 460]. Thus, the behavior seen in the elevated platform test may reflect heightened avoidance learning in PNE-exposed animals. Nevertheless, because the behavior observed in the Suok test mirrors that seen in the elevated platform test, the study design does not alter the interpretation that PNE exposure results in long-term effects on anxiety.

Litter effects

It is important to consider the effects of individual maternal differences on behavioral outcomes. Previous work in our laboratory has confirmed similar food intake, water intake, and weight gain between experimental and control pregnant dams [52]. However, aspects of maternal care have previously been found to modify behavioral responses [461, 462], and genetic differences between different litters may

influence behavior as well. Indeed, nicotine may differentially modify maternal care behaviors, which in turn could affect behavioral responses between litters. Current analyses suggest no consistent litter effects. Specifically, analyses controlling for litter effects, using the litter rather than the offspring as the unit of analysis, confirm our findings in individual offspring. Thus, we feel confident that prenatal nicotine exposure disrupts some aspects of sensory and motor function and increases anxiety-related behaviors in mice.

Brain and body weights at 6 months of age are not affected by gestational nicotine treatment

Reductions in newborn brain weights as a result of prenatal nicotine exposure have previously been observed in this mouse model [52]. Low brain weights early in development are thought to be caused by the targeting of mitosis pathways by acute doses of nicotine, resulting in the premature cessation of proliferation and onset of differentiation [40, 422]. Recovery of brain weights in prenatally-exposed offspring has been well documented, with normal brain weight being attained as early as P10 and maintained until P50 [45, 52, 397, 405]. Brain weight comparisons between control and PNE offspring late in adulthood have not been previously documented. Consistent with data from younger animals, analyses of measurements in brain weight in this study reveal no differences between 6-month-old control and experimental mice.

This study also investigated differences in body weights between control and nicotine exposed animals. Prenatal nicotine exposure is thought to result in intrauterine growth retardation (IUGR) via decreases in nutrient diffusion into placental circulation [47, 53]. Indeed, numerous studies in humans and animals have found an association between prenatal nicotine or tobacco exposure and decreased body weights at birth [67,

418, 463]. Previous findings from our laboratory on this mouse model revealed reduced pup weight in newborn PNE animals; however this effect is rescued by post-natal day (P) 10 [52]. Similarly, decreased body weights of human children prenatally exposed to tobacco have been shown to recover over time [464]. Consistent with these findings, body weights for control and nicotine animals at six months of age were not found to be significantly different. Low birth weights in humans have been associated with cognitive and behavioral differences later in development [421]. Thus, low birth weights in this PNE model may translate to behavioral deficits that were observed at six months of age.

Cortical size in 6-month-old mice is not altered by prenatal exposure to nicotine

Cortical widths and lengths in hemispheres of control and PNE animals were recorded. Previous measurements in this mouse model demonstrated reductions in brain lengths of newborn PNE mice, an effect that recovered by P10 [52]. The current study reveals no significant differences in cortical lengths and widths between control and PNE brains of 6-month-old mice, confirming that PNE mice maintain normal recovered cortical sizes throughout adulthood.

Cortical thicknesses of sensory and motor areas are not affected by prenatal exposure to nicotine in 6-month-old mice

Given the association between decreased sensory and motor function and prenatal tobacco exposure in humans, we hypothesized that alterations in sensory and motor regions of the cerebral cortex may result from exposure to prenatal nicotine. Although previous analyses of P0 intraneocortical connectivity in putative somatosensory and visual regions showed no alterations as a result of prenatal exposure [52], other anatomical differences in the cortex have been documented in other

laboratories. Thickness of hippocampal cell layer CA3 was reduced in young animals (P21, P30) prenatally exposed to nicotine [465]. Human female adolescents exposed to tobacco display reductions of cortical thickness in orbitofrontal, parahippocampal, and middle frontal cortices [441], and researchers have found a global thinning of the cortex in children with ADHD, a condition that has been associated with maternal smoking [14, 466]). Roy and Sabherwal (1994) found decreases in somatosensory cortical thicknesses of rats prenatally exposed to nicotine until P20. This effect was accompanied by a reduction in cortical cell size and decreased dendritic branching in layer 5 pyramidal cells in rat somatosensory cortex up to P40. The current study sought to assess cortical thickness in three sensory and motor areas (putative motor, somatosensory, and visual cortices) in older 6-month-old mice prenatally exposed to nicotine. As cortical thickness in children with developmental disabilities has been linked with behavioral outcomes [466], this study attempts to bridge observed long-term behavioral differences with anatomical alterations. Gestational nicotine exposure did not alter measures of sensory and motor cortical thickness in six-month old mice, suggesting that prenatal nicotine is not exerting its behavioral effects through gross anatomical changes in the cortex that are maintained throughout adulthood.

Dangers of Maternal Smoking and Nicotine Replacement Therapy (NRT)

The current study underscores the importance of informing pregnant women of the dangers of smoking and using NRTs during pregnancy. Although approximately 75% of pregnant smokers report the desire to quit smoking during pregnancy, less than a third successfully abstain, and 15% relapse within 6 months [33, 467]. Our results implicate nicotine as a substance that results in clear long-term behavioral deficits when

administered prenatally. NRTs, although considered a safer alternative to cigarette smoke, represent a danger to the fetus and are classified as Pregnancy Category C and D drugs [35, 36].

CONCLUSIONS

Our laboratory has previously found significant reductions in body and brain weights at birth in a mouse model of PNE, implicating nicotine, the main psychoactive ingredient of tobacco, as a teratogenic substance [52]. The current study investigated the long-term effects of prenatal nicotine exposure on sensory and motor function, anxiety-related behaviors as well as brain and body weight, and cortical size and thickness in 6-month-old female mice. Our results reveal that prenatal nicotine exposure results in decreased sensorimotor integration and increased anxiety in exposed mice in the absence of gross neuroanatomical changes in the cerebral cortex, demonstrating the long-term behavioral teratogenicity of nicotine. This study suggests that nicotine replacement therapy is not a safe alternative for use during pregnancy. Health care providers are therefore advised to counsel pregnant women on the dangers of nicotine exposure to the fetus.

GENERAL CONCLUSIONS

In all, this dissertation provides an analysis and review of the prevalence and effects of maternal consumption of environmental toxins, and will hopefully contribute to the prevention and understanding of developmental disorders and physiological deficits, the likes of which have grave implications for public health and productivity.

Healthy post-pregnancy outcomes are contingent upon an informed regimen of prenatal care encouraging healthy maternal consumption habits. Thus, this dissertation first examines the consumption habits of pregnant women in respect to the frequency at which potentially teratogenic substances were consumed. Occurrences and frequencies of maternal intake of healthy foods and beverages, unhealthy foods, unhealthy beverages, and medication in a population of predominantly Hispanic women in Southern California were assessed by way of the Food, Beverage, and Medication Intake Questionnaire (FBMIQ), in the hopes of shedding light on the prevalence of risky consumption habits. The described study reveals widespread consumption of fresh fruit, meats, milk and juice and indicate most women used prenatal vitamin supplements, yet show that a proportion of pregnant women consumed BPA, MeHg, caffeine, alcohol, and certain medications at varied levels during pregnancy.

Smoking during pregnancy represents a significant risk to the unborn fetus, and may impart various physiological, cognitive, and behavioral postnatal effects. This dissertation describes research in which a PNE mouse model was developed to investigate physiological and behavioral changes. CD-1 dams were given

injections of nicotine or control vehicle throughout gestation and their offspring were raised to birth, postnatal day (P) 0, 10, 20, 50, and 6 months of age. Dam nutrition, dam weight gain and litter size were not found to be significantly altered by nicotine treatment. Given the role of the mammalian neocortex in governing sensory integration and higher-order processes such as emotion, cognition and behaviors, our examination sought to analyze the aspects of the brain and neocortex that could possibly underly some of the cognitive and behavioral phenotypes of prenatal nicotine exposure (PNE). Thus, our analyses included postnatal measurements of brain weight, brain widths and lengths, development of neocortical circuitry, and cortical thickness measures. Exposed mice were found to exhibit reduced brain and body weights at birth, a phenotype that recovered by postnatal day 10. Analysis of brain and body weights through six months of age revealed no significant differences between control and exposed offspring. No changes in neocortical circuitry or thickness in sensory and motor areas were found.

Additionally, this dissertation describes persistent behavioral effects of PNE on sensory and motor function and anxiety measures in female mice at six months of age. This is the first study to investigate PNE-induced behavioral alterations in mice aged well into adulthood. These mice underwent a battery of tests including the Suok test (assaying sensorimotor integration, hyperactivity, anxiety, and exploration), the elevated platform test (assaying measures of anxiety-mediated exploration), and the elevated plus maze (assaying anxiety). Female mice exposed to prenatal nicotine exhibited decreased likelihood of jumping during the elevated platform test and decreased directed exploration in

the Suok test, both indicative of increased anxiety. These mice were also characterized by an increased number of missteps on the Suok test, indicative of deficits in sensorimotor integration abilities. Such analyses describe immediate and long-lasting postnatal effects of prenatal nicotine exposure, underscoring the importance of abstaining from nicotine or tobacco products during pregnancy.

There exists a plethora of research conducted on the detrimental effects of prenatal exposure to environmental toxins, which have widespread effects on brain and body systems. The developing system in particular is more susceptible to toxic insults, and small changes in utero can result in persistent, long term deficits. Pregnant women who consume addictive substances, like nicotine, or pollutant substances are at risk for unhealthy post-pregnancy outcomes . This dissertation reviews at length the potential teratogenicity of nicotine, alcohol, caffeine, MeHg, PCBs, BPA, and tap water contaminants, so as to characterize the current body of literature detailing the effects and implications of prenatal exposure to environmental toxins. Hopefully, the works detailed in this dissertation will provide a foundation upon which future researchers can build a better understanding of how prenatal exposures contribute to harmful developmental deficits.

ACKNOWLEDGEMENTS: The text of this dissertation is in part a reprint of material as it appears in *Neuroscience Research* V73, I4 (282-291), *Neuroscience Research* V79, I4 (41-51), and *Nutrition Journal* V12, I1 (91-115). Dr. Kelly Huffman, an author on these publications, guided and supervised the direction of this research.

TABLES AND FIGURES

TABLES

Table 1.1. Demographic information of respondents

Age (<i>n</i> = 194)	% Preg women	Education (<i>n</i> = 191)	% Preg women
18-20	7.7	Elementary	0.5
21-25	20.1	Middle school	3.7
26-29	27.3	High school	27.7
30-35	34	Some college	41.9
36-39	8.2	College degree	17.8
40+	2.6	Graduate degree	8.4
Race (<i>n</i> = 190)	% Preg women	Income (<i>n</i> = 171)	% Preg women
White	2.1	0-25K	35.1
Hispanic	87.4	25,001K - 50K	33.9
African American	4.7	50,001K-75K	17.5
Asian/Pacific Islander	4.2	75,001K-100K	11.1
Middle-eastern	0.5	100K+	2.3
Other	1.1		

TABLE 1.1. Sample characteristics of respondents. Values for Age, Education, Race, and Income are listed as percentages for number of pregnant women. (n = 171-194).

Table 1.2. Food consumption habits

Food Consumed	% Preg women
Any meat	99.5
Chicken	98.5
Beef	84
Pork	52
Any fish	73.9
Tuna	52
Talapia	34.2
Salmon	25.5
Other fish	19.9
Fresh fruit	100
Bananas	95.4
Oranges	88.8
Apples	88.3
Other fresh fruit	40.3
Any canned foods	73.9
Canned fruits/veggies	52.3
Canned soup	41.6
Canned tuna	41.6
Sugary desserts	97.5
Ice cream	82.7
Baked desserts	70.1
Chocolate	65
Other desserts	3.6
Fast foods	96
Burgers	85.2
French Fries	77.9
Chicken products	53.6
Other fast foods	7.7

TABLE 1.2. Number of women who reported consuming a given food during pregnancy. Values are listed as percentages for number of pregnant women. (n = 195-200).

Table 1.3. Beverage consumption habits	
Beverage Consumed	% Preg women
Any water	100
Bottled water	96
Home filtered water	36.9
Tap water	12.1
Other water	1.5
Any milk	95.4
Lowfat	79
Whole	20.9
Other milk	9.2
Organic	6.7
Skim	4.1
Any juice	94.9
Orange juice	76.8
Apple juice	69.1
Juice blends	41.2
Other juice	12.9
Any caffeine	80.1
Colas	60.2
Coffee	45.5
Tea	29.8
Other caffeinated beverage	2.1
Energy Drinks	1.5
Decaffeinated beverages	22.5
Any alcohol	5.8
Beer	1.6
Wine	4.7
Mixed drinks	1.1
Shots/liquor	0.5

TABLE 1.3. Number of women who reported consuming a given beverage during pregnancy. Values are listed as percentages for number of pregnant women. (n = 188-199).

Table 1.4. Medication/ vitamin consumption	
Substance Consumed	% Preg women
Over-the-counter Meds	45.3
Acetaminophen	38
Cough/ Cold Meds	4.2
Ibuprofen	3.1
Aspirin	1.6
Decongestants	1.6
Prescription Meds	84
Prenatal Vitamins	83.4
Morning Sickness Medications	8.8
Pain Medications	3.6
Antidepressants	0

TABLE 1.4. Number of women who reported consuming vitamins or medications during pregnancy. Values are listed as percentages for number of pregnant women. (n = 192-194).

Table 1.5. Frequency and trimester of consumption

Substance Consumed	Frequency (displayed as % of women reporting intake)					Trimester (displayed as % of women reporting intake)						
	1-3 entire preg.	1-3/month	1-3/week	4-6/week	7+/week	1	2	3	1,2,3	1,2	2,3	1,3
Food												
Meat	52	12.9	51.5	22.2	8.2	9.9	9.4	3.6	66.1	2.6	7.8	0.5
Fish	41.4	38.6	16.6	2.8	0.7	12.3	27.5	8.7	31.2	4.3	14.5	1.4
Fruit	2.6	3.6	28	34.7	31.1	6.3	4.2	1.6	77	3.1	7.3	0.5
Canned foods	28	31.5	28.7	9.8	2.1	16.5	11.5	5	48.9	6.5	10.1	1.4
Sugary desserts	10.6	39.2	37	10.1	3.2	8.1	14	9.7	53.2	2.2	12.9	0
Fast foods	19.1	47.5	25.7	5.5	2.2	8.9	15.1	10.1	43	7.8	15.1	0
Beverage												
Water	1.5	0.5	3.1	6.7	88.2	5.8	3.2	0.5	85.8	2.1	2.1	0.5
Milk	2.2	8.2	23	35.5	31.1	6	4.4	1.1	71.4	4.9	11.5	0.5
Juice	2.8	11.7	35.8	30.7	19	7.4	6.3	2.8	68.8	6.3	8	0.6
Caffeine	14	30.7	40.7	11.3	3.3	10.9	15.4	8.3	40.4	5.1	18.6	1.3
Beer / wine	88.9	11.1	0	0	0	50	30	10	0	10	0	0
Mixed drinks/liquor	100	0	0	0	0	100	0	0	0	0	0	0
Medication												
Over-the-counter	53.7	31.3	7.5	3	4.5	19.3	21.7	13.3	28.9	6	7.2	3.6
Prescription	7.1	5.3	1.8	8.8	76.1	10.9	2	2	72.8	6.1	4.1	2

TABLE 1.5. Frequency of consumption for foods, beverages, and medications and with trimester for which frequency data is applicable. All values are percentages of women who reported intake of a given item. Frequency values are listed as percentages for number of women who reported consuming a given item 1-3 times during their entire pregnancy, 1-3 times per month, 1-3 times per week, 4-6 times per week, or 7 or more times per week (n = 10-194). Trimester values are listed as percentages for number of women who reported consuming a given item during the first, second, or third trimester only, all three trimesters, or two of the three trimesters. (n=10-200).

FIGURES

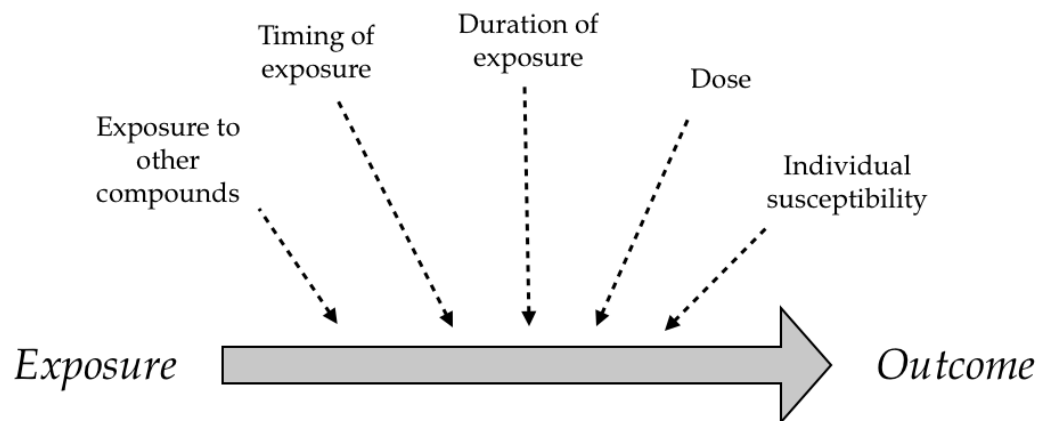


FIGURE 1.1. The severity and pattern of teratogenic effects depend upon a variety of factors including extent of exposure to other compounds, timing of exposure, duration of exposure, dosage, and individual susceptibility.

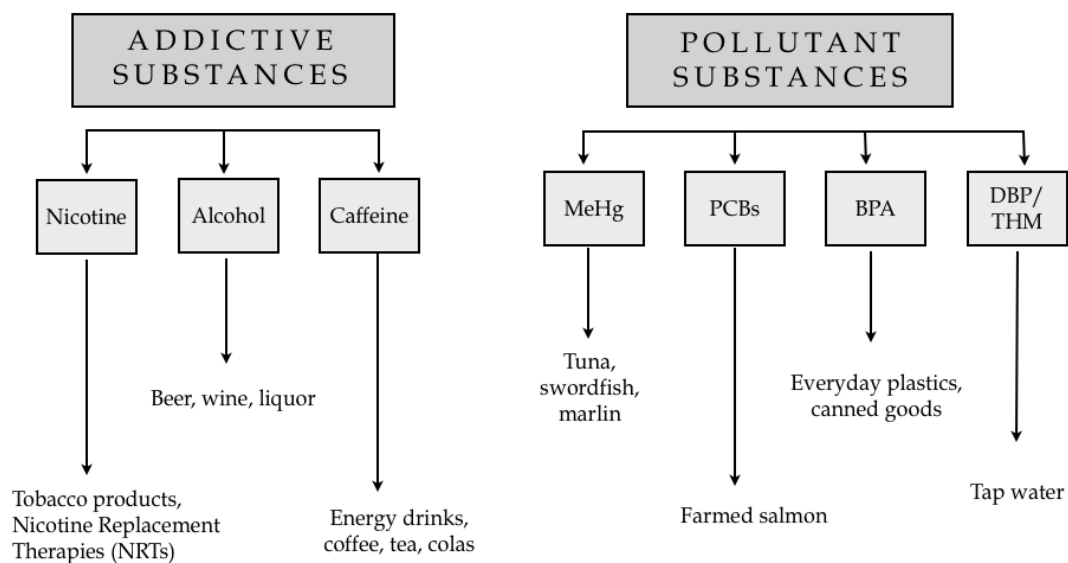


FIGURE 2.1. Environmental substances with potentially teratogenic effects. Addictive substances reviewed include nicotine, alcohol, and caffeine, which can be found in a number of commonly ingested products. Pollutant substances include methylmercury (MeHg) and polychlorinated biphenyls (PCBs), which can be ingested by consuming certain types of seafood, Bisphenol-A (BPA), which can leech from everyday encountered plastics and canned foods, and tap water contaminants, including disinfection byproducts (DBPs) and trihalomethanes (THMs).

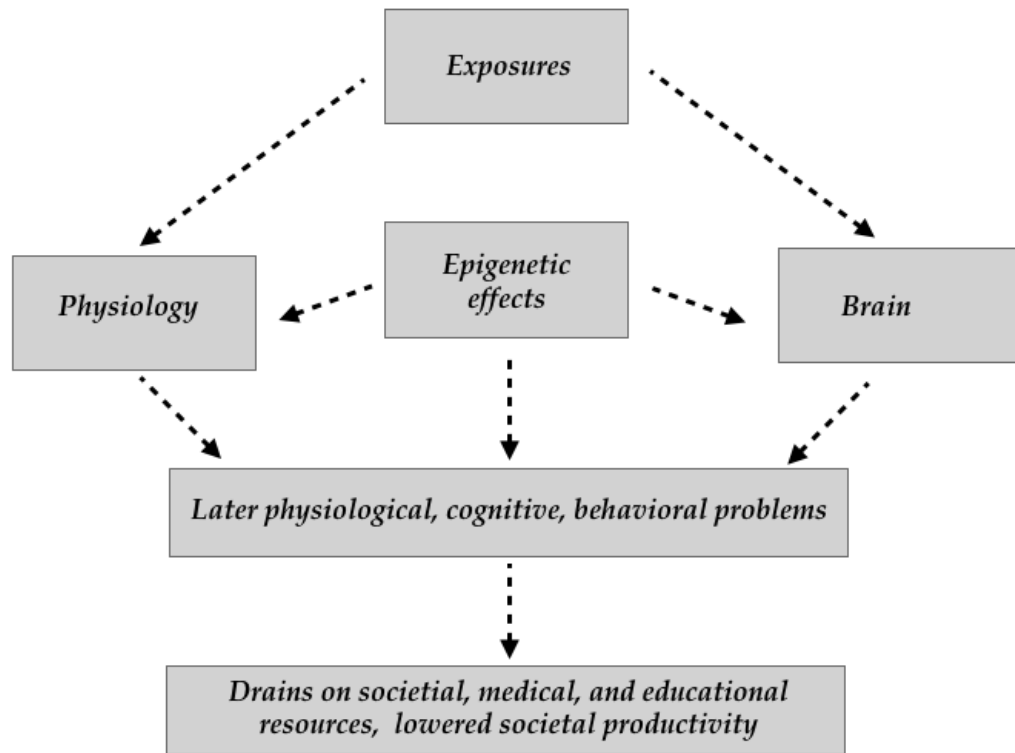


FIGURE 2.2. Prenatal exposures may affect the brain, impact physiology, or lead to alterations on the epigenome. Such subclinical or serious changes can lead to later problems with health, mental health, behavior, exhausting societal, medicinal, and educational resources while lowering societal productivity.

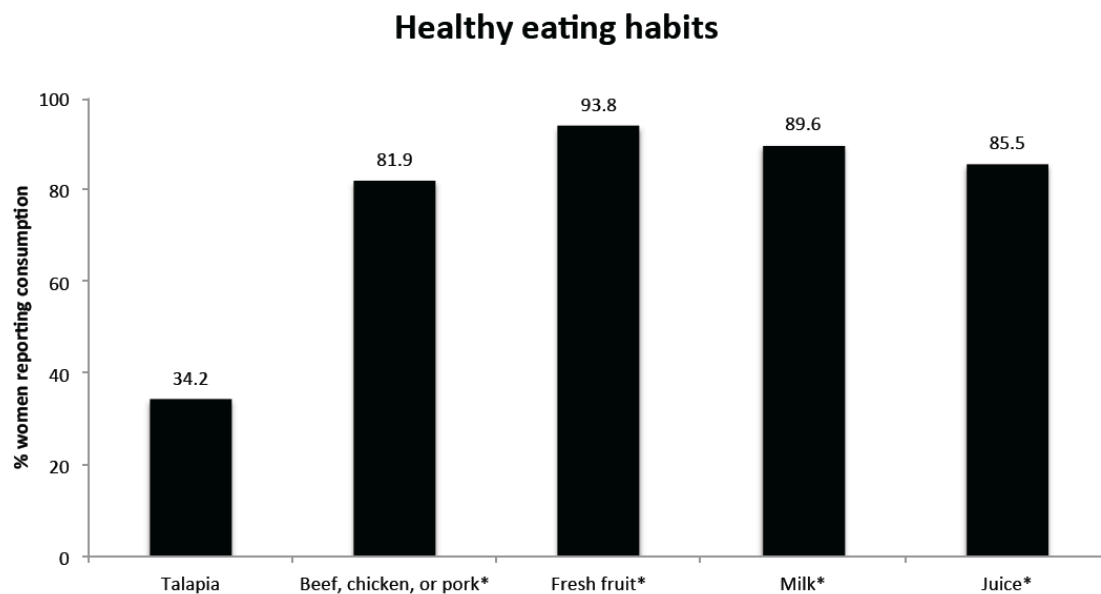


FIGURE 3.1. Graphical depiction of percentages of women who reported eating healthy foods during their pregnancies. Asterisks indicate percentages of women who ate or drank a given food or beverage at least once per week. (n = 196-200).

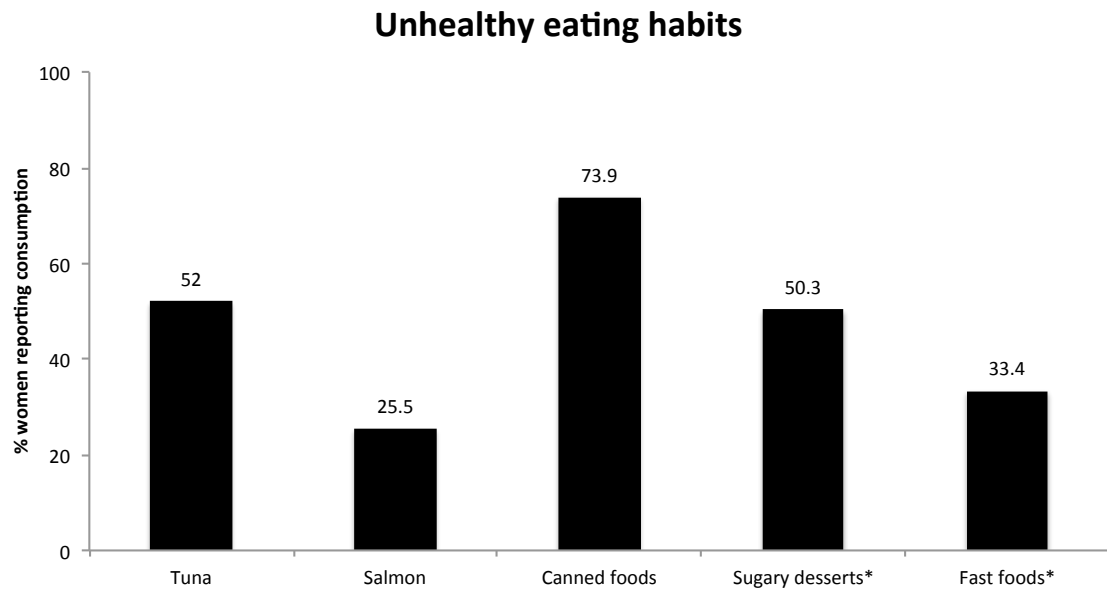


FIGURE 3.2. Graphical depiction of percentages of women who reported eating healthy foods during their pregnancies. Asterisks indicate percentages of women who ate or drank a given food or beverage at least once per week. (n = 196-200).

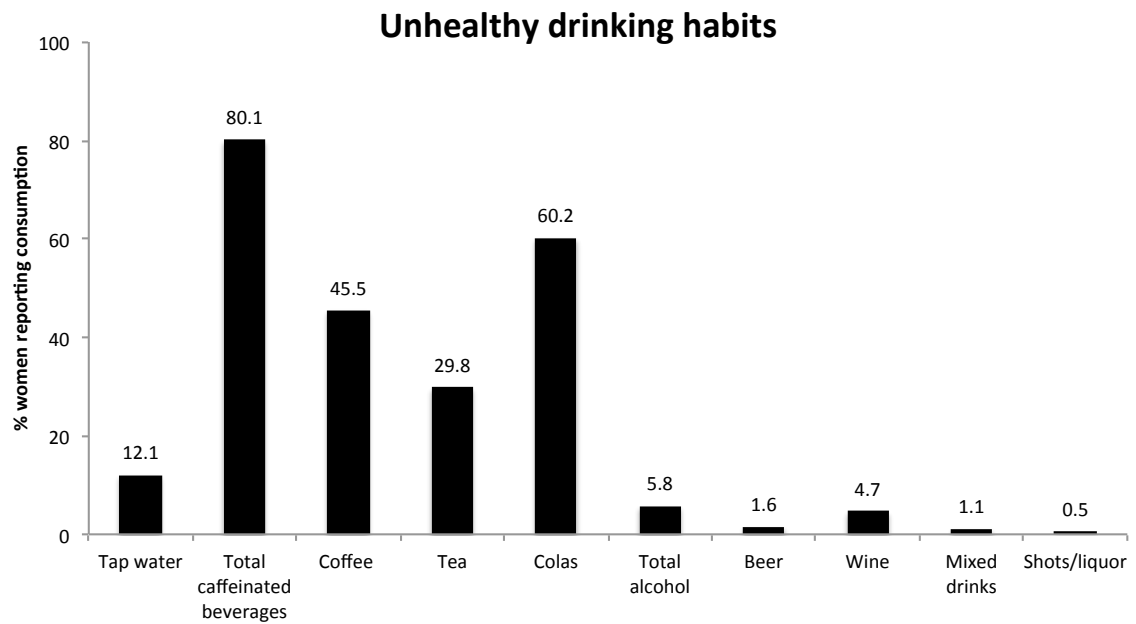


FIGURE 3.3. Graphical depiction of percentages of women who reported eating healthy foods during their pregnancies. Asterisks indicate percentages of women who ate or drank a given food or beverage at least once per week. (n = 196-200).

Average Daily Dam Weight Gain, Intake, and Litter Size

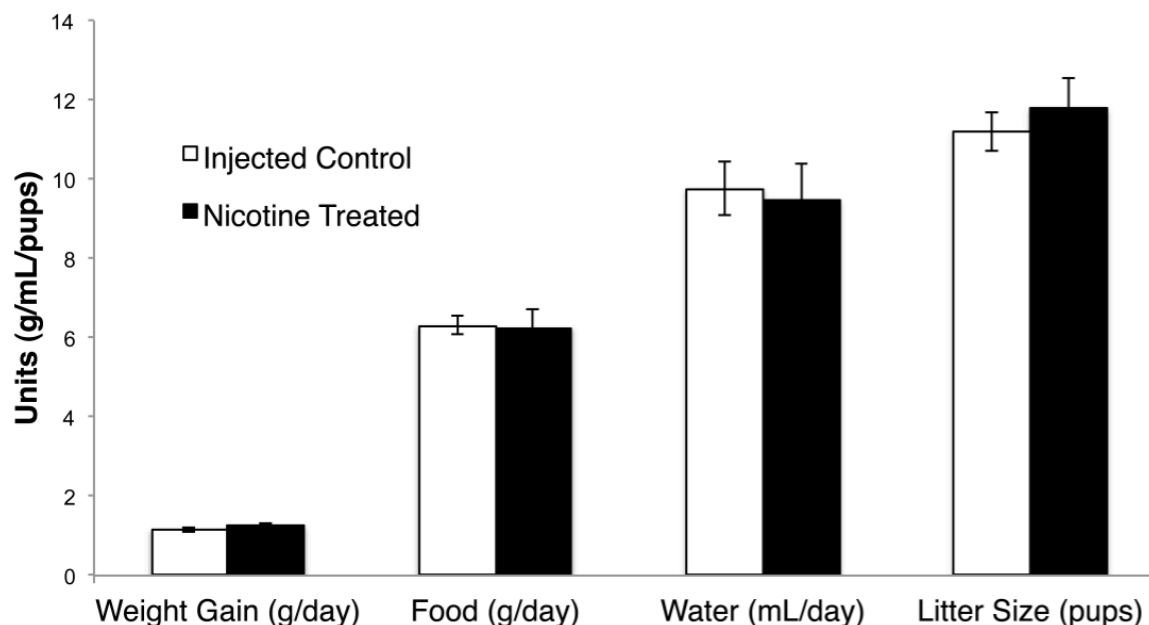


FIGURE 4.1. *Average daily maternal weight gain, food intake, water intake, and litter size.* Average daily weight gain, food intake, and water intake in dams were calculated from measurements recorded daily from gestational day 0.5 - 17.5. No significant differences between control and nicotine treated groups were found between average daily weight gains, food intake, or water intake of nicotine treated and injected control groups ($n = 4-6$ per group; t -test; $p < 0.05$). Litter sizes of dams in nicotine treated and injected control groups were noted at birth. No significant differences were found (t -test, $n = 5$ per group). Data is expressed as group means \pm S.E.M.

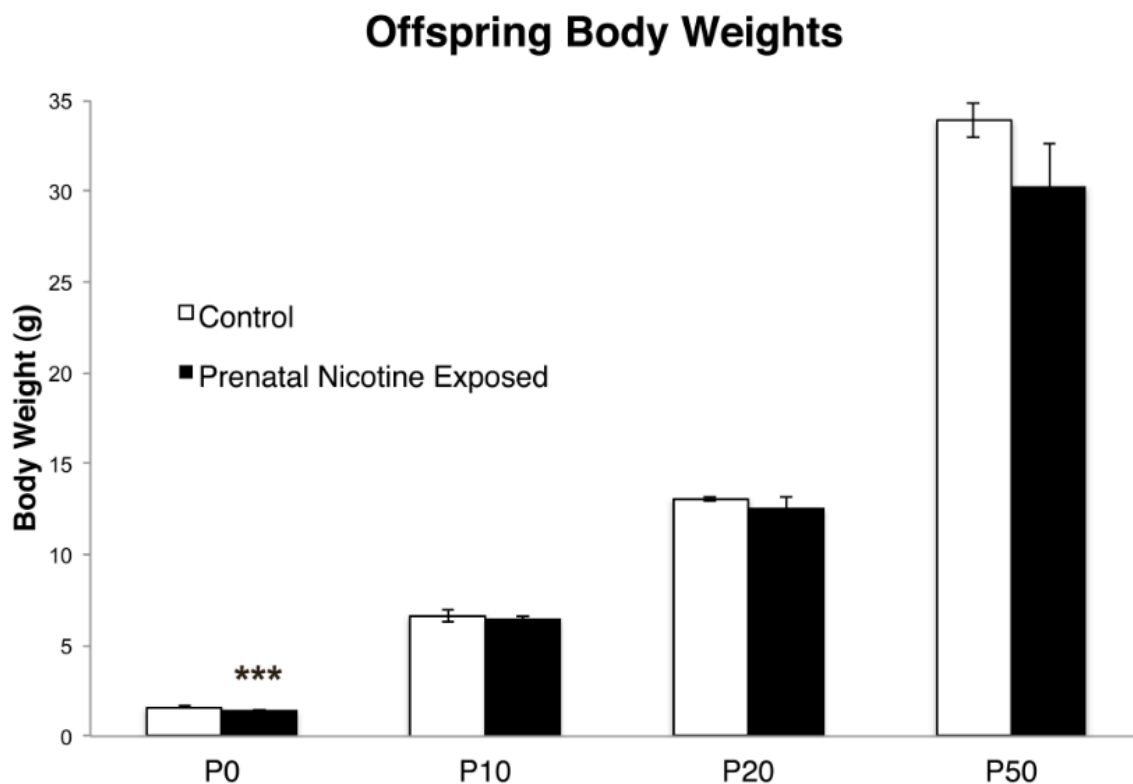


FIGURE 4.2. *Offspring body weights at postnatal day (P) 0, P10, P20, and P50.*

Omnibus ANOVA (treatment \times age) revealed main effects of age ($F(3,75)=767.53$, $p<0.001$) and treatment ($F(1,75)=10.34$, $p<0.05$) as well as an age by treatment interaction ($F(3,75)=10.05$, $p<0.05$). Average body weight of P0 prenatal nicotine exposed offspring ($n=10$) was significantly lower than that of P0 control offspring ($n = 12$; post-hoc t-test; *** $p<0.001$). No significant differences were found between experimental and control groups at P10 ($n = 10$ per group), P20 ($n=10$, $n = 6$), or P50 ($n=8$, $n=10$; post-hoc t-test $p>0.05$). Data is expressed as group means \pm S.E.M.

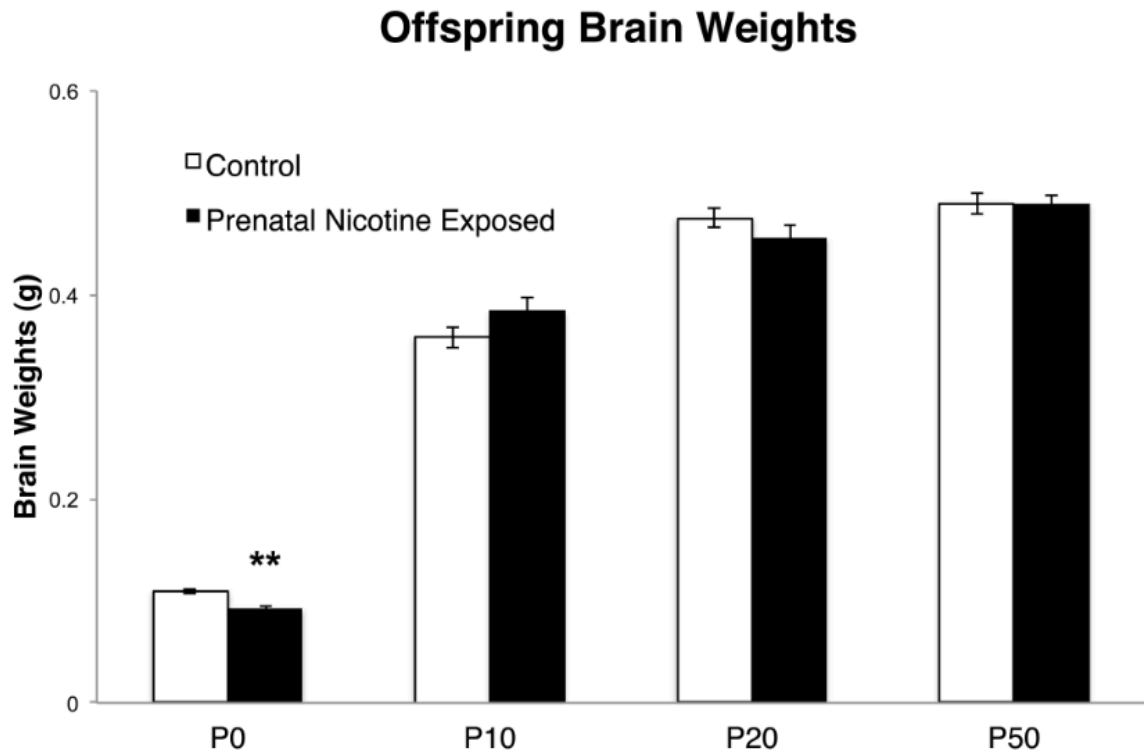


FIGURE 4.3. *Offspring brain weights at postnatal day (P) 0, P10, P20, and P50.*

Omnibus ANOVA (treatment x age) revealed a main effect of age ($F(3, 79)=745.23$, $p < 0.001$) and an interaction effect of age and treatment ($F(3, 79)=2.442$, $p=0.035$). Significant differences in brain weights were found between control and prenatal exposed nicotine offspring on P0 ($n = 10$; post-hoc t-test; $**p < 0.01$). At P10, P20, and P50, no significant differences in brain weights were found between control and nicotine exposed offspring ($n = 10$ for all groups; post-hoc t-test; $p > 0.05$). Data is expressed as group means \pm S.E.M.

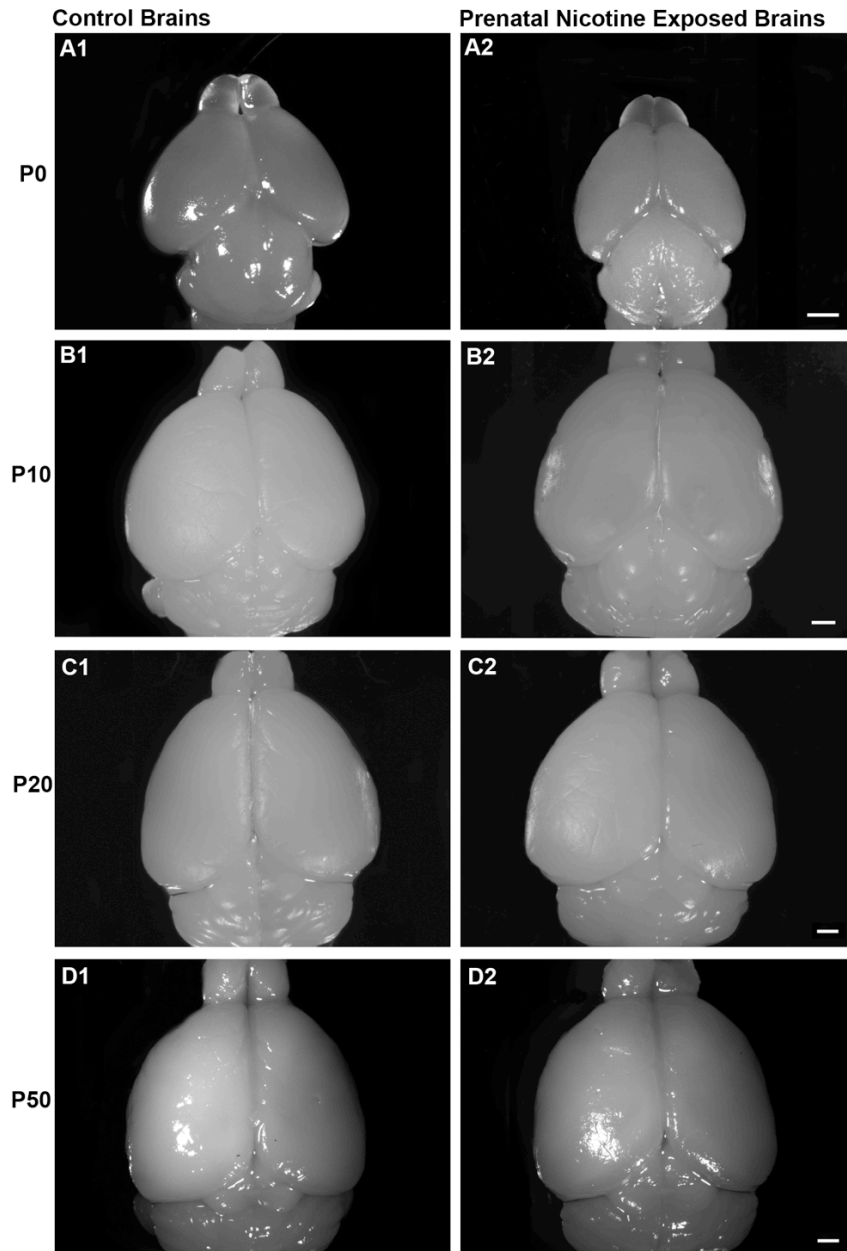


FIGURE 4.4. *Dorsal views of control and prenatal nicotine exposed brains for size comparison.*

At P0, control brains (A1) were noticeably larger than prenatal nicotine exposed brains (A2; P0 scale= 1 mm). However, at P10, P20, and P50, no differences in overall brain size were observed between control (B1, C1, D1) and prenatal nicotine exposed (B2, C2, D2) brains (P10 - P50 scale= 500 μ m). Rostral is up and lateral to the left and right.

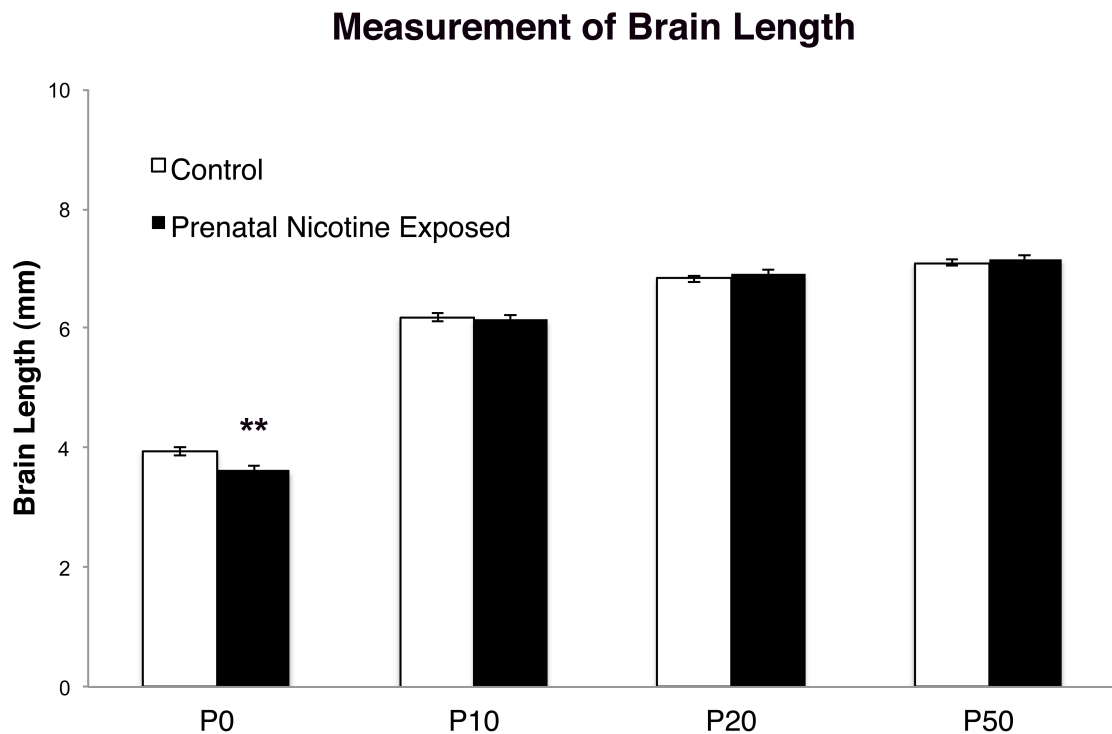


FIGURE 4.5 . Offspring brain lengths at postnatal day (P) 0, P10, P20, and P50.

Omnibus ANOVAs revealed significant main effects of age on lengths of P0 x P10 brains ($F(1,39) = 1082.41$; $p < 0.001$). An interaction effect of treatment and age was found in P0 x P10 brain lengths ($F(1,39) = 4.294$; $p < 0.05$). Post-hoc analyses confirmed differences in brain lengths between control and prenatal exposed nicotine offspring on P0 ($n=10$; post-hoc t-test; $**p < 0.01$). At P10, P20, and P50 ($n=10$ for all groups), no significant differences in brain lengths were found between control and nicotine exposed offspring. Data is expressed as group means \pm S.E.M.

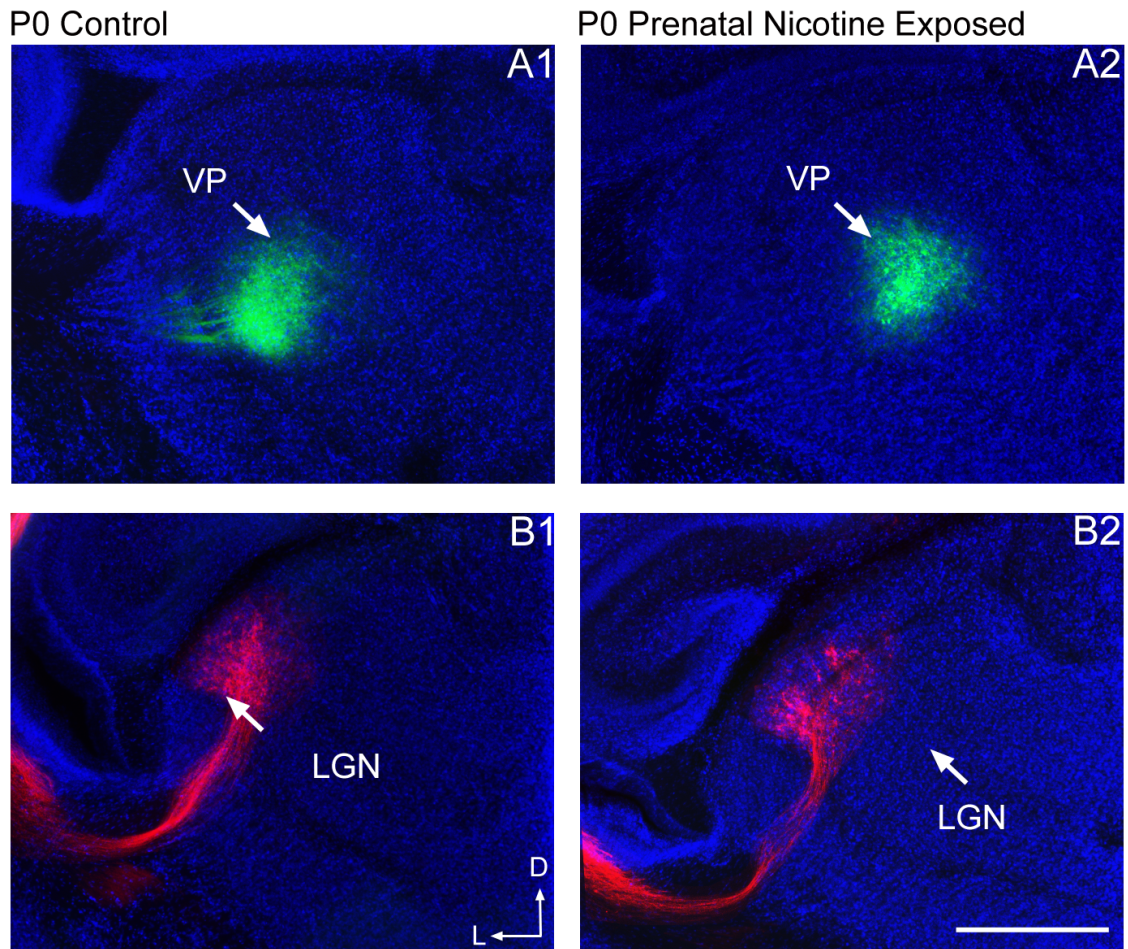


FIGURE 4.6. *Comparison of P0 control and PNE TCAs by way of retrogradely labeled cells in dorsal thalamic nuclei.*

Coronal sections through the thalamus from control brains (A1, B1) and nicotine exposed brains (A2, B2) are presented. Arrows indicate: labeled cells in the ventral posterior nucleus (VP; A1, A2), resulting from putative somatosensory cortex DPLs, and labeled cells in the lateral geniculate nucleus (LGN; B1, B2), resulting from putative visual cortex DPLs. No differences in the dorsal thalamic nuclear locations of retrogradely labeled cells were observed between control and prenatal nicotine exposed brains. Scale= 500 μ m; sections are presented with dorsal (D) up and lateral (L) to the left. (n=5 per group)

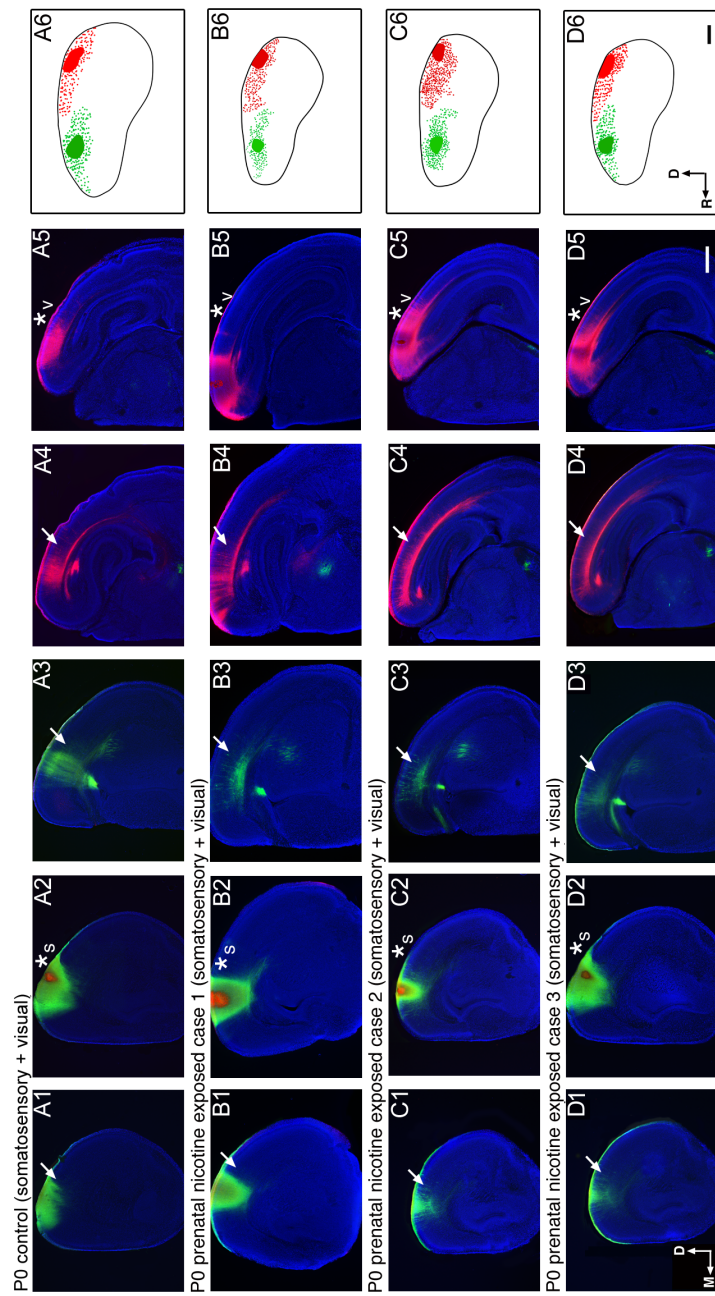


FIGURE 4.7. Comparison of putative somatosensory and visual cortex INCs in P0 control and PNE brains.

Rostral to caudal series of coronal sections showing retrograde cell labeling after DiA (green) or DiI (red) dye crystal placements into putative somatosensory and visual cortices, with reconstructions of labeled cell body locations in flattened neocortex at P0. 100 μ m sections of hemispheres from a representative control brain (A1-A5) and two representative nicotine exposed brains (B1-B5, C1-C5) are shown. The dye placement locations (DPLs) are indicated by asterisks in putative somatosensory cortices (A2, B2, C2), and visual cortices (A5, B5, C5). In both control and PNE sections, retrogradely labeled cells were located rostral and caudal to the somatosensory DPL (arrows, A1 and A3, B1 and B3, C1 and C3), and both rostral (arrows, A4, B4, C4) and medial to the visual DPL. Reconstructions of labeled cell bodies were analyzed and revealed no differences in INC location between control and experimental groups in putative somatosensory and visual cortices (A6, B6, C6). Scale bars: 500 μ m; D, dorsal; M, medial; R, rostral; small dots in A6, B6, and C6 indicate retrogradely labeled cell bodies from the somatosensory DPL (green) and visual DPL (red). Larger circles indicate the extent of the somatosensory DPL (green) and the visual DPL (red).

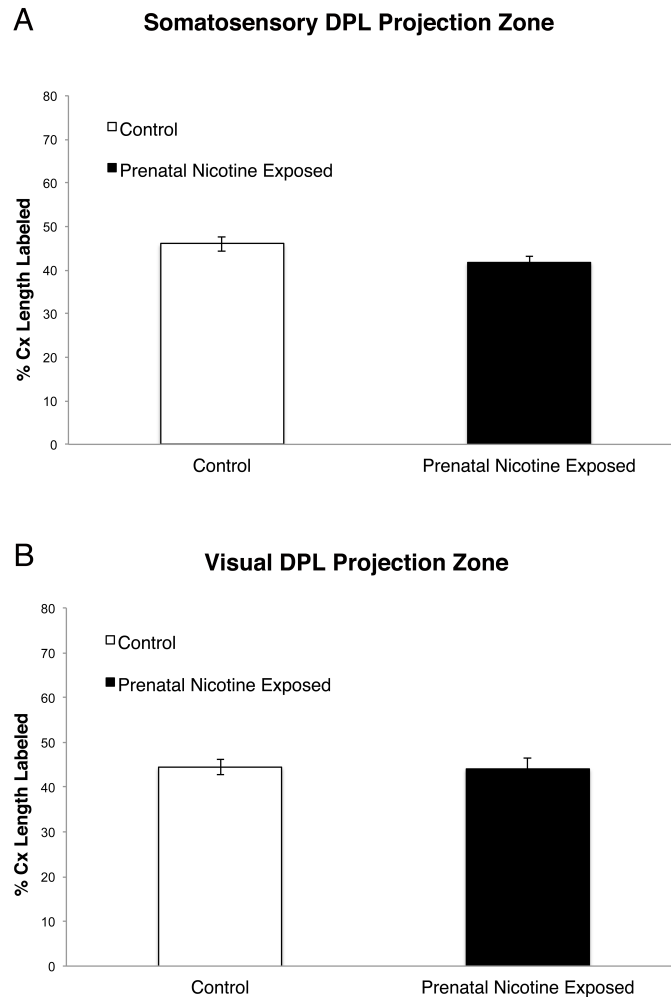


FIGURE 4.8. *Analysis of projection zones from DPLs in somatosensory and visual cortices in control and PNE brains.*

Projection zones of somatosensory and visual cortices were measured in reconstructed INC maps of control and PNE brains at P0. Extent of cell labeling from DPLs in somatosensory cortex and visual cortex along the rostral-caudal axis of reconstructed maps was taken as a percentage of the length of the neocortex. Comparison of percentage of cortex length labeled from DPLs in somatosensory (A) and visual (B) cortices of control and PNE brains revealed no significant differences ($p > 0.05$; t-test; $n = 5$ per group). Data is expressed as percentages of cortical length labeled \pm S.E.M.

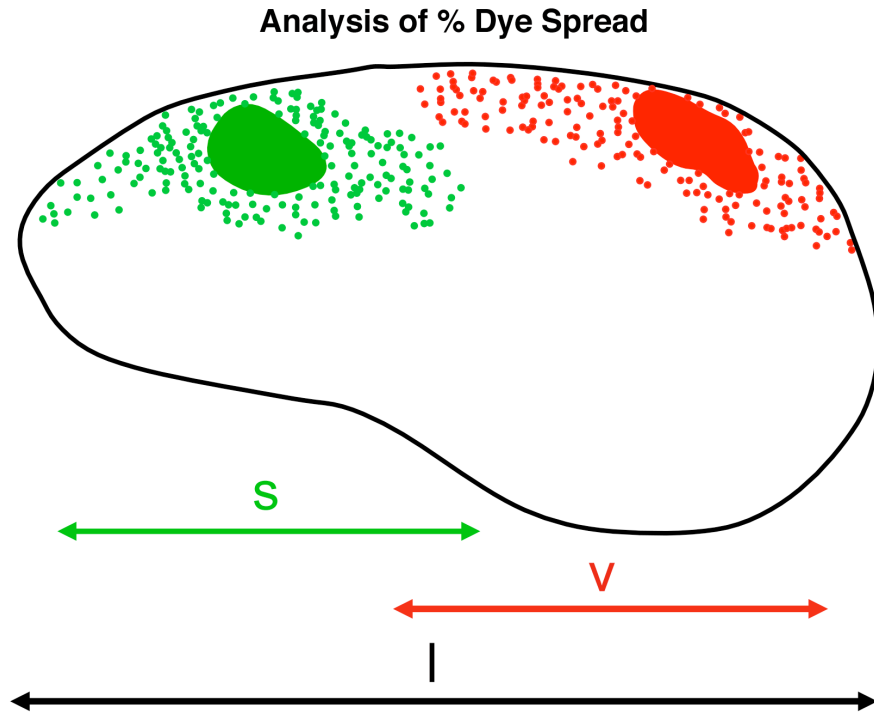


FIGURE 4.9. *Analysis of projection zones from DPLs in somatosensory and visual cortices.* Extent of cell labeling from DPLs in somatosensory cortex (S; green double-sided arrow) and visual cortex (V; red double-sided arrow) were measured along the rostral-caudal axis of reconstructed maps. Measurements were expressed as a percentage of the length (L; black double-sided arrow) of the neocortex. D, dorsal; R, rostral.

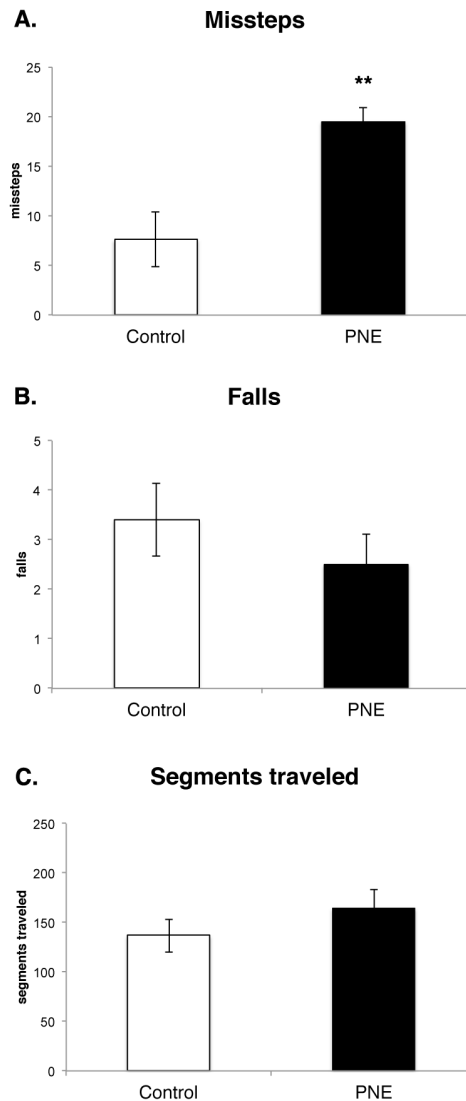
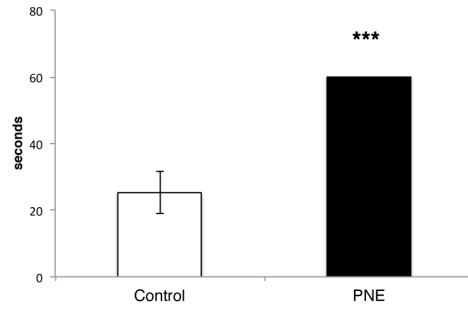


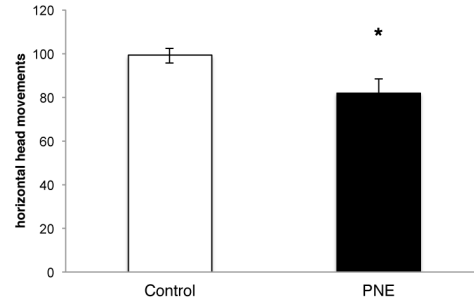
FIGURE 5.1. Behavioral measures of sensory and motor function in 6-month-old control and PNE mice.

Sensory and motor function was assessed with the Suok test. Mice prenatally exposed to nicotine (PNE) exhibited significantly more missteps during the Suok assessment as compared to control mice (A; t-test, ** $p=0.00205$). The number of falls for each mouse was recorded during the 5 minute Suok test. The groups did not significantly differ (B; t-test; $p=0.335$). No significant differences were found between groups in number of segments traveled during the Suok test (C; t-test, $p=0.290$). Control, $n = 8$; PNE, $n = 10$; Data is expressed as group means \pm S.E.M.

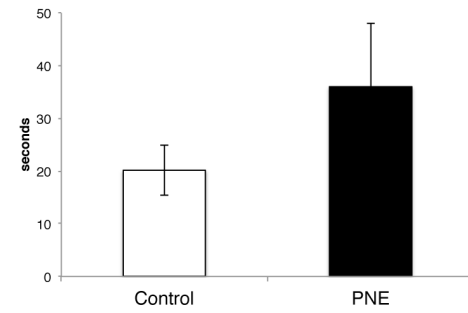
A. Platform test



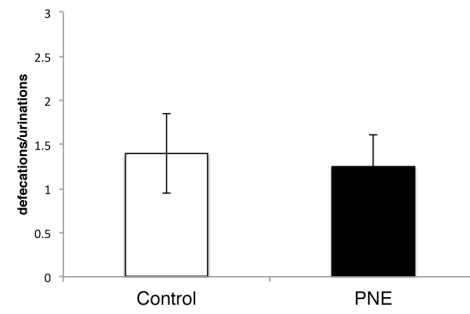
B. Directed exploration



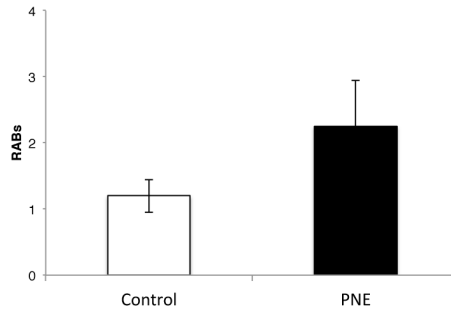
C. Latency to leave central zone



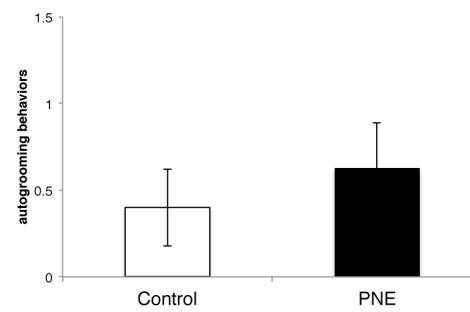
D. Defecation/urination



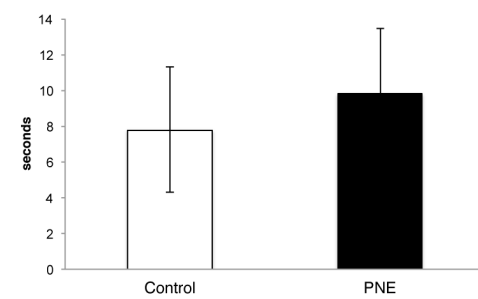
E. Risk assessment behavior



F. Autogroom



G. Time spent in open arms



H. Time spent in closed arms

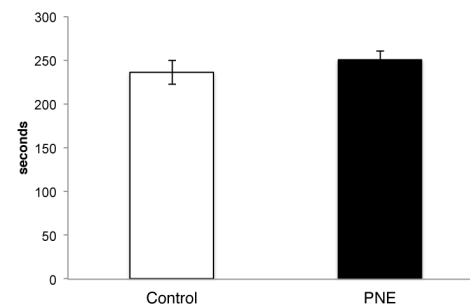
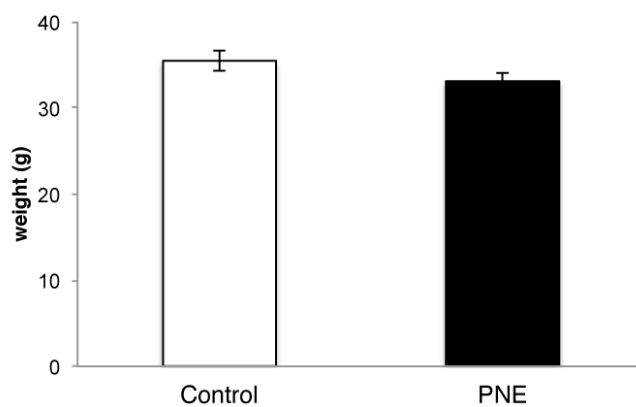


FIGURE 5.2. *Behavioral measures of anxiety in 6-month-old control and PNE mice.*

Anxiety levels of 6-month-old control and PNE mice were assessed using the elevated platform test, the Suok test, and the elevated plus maze test. While PNE mice failed to move from the elevated platform, control mice remained atop the platform for a significantly shorter period of time (A; t-test, *** $p = 0.000371$). PNE mice also displayed significantly less directed exploration than control mice (B; t-test, * $p=0.0441$). Groups did not significantly differ in average latency to leave the central zone (C; t-test, $p=0.255$), number of urinations and defecations (D; t-test, $p=0.692$), incidences of RABs (E; t-test, $p=0.193$), and autogroom instances (F; t-test, $p=0.525$; For all tests: control, $n = 8$; PNE, $n = 10$). The time spent in open and closed arms was recorded during a 5-minute elevated plus maze. The groups did not significantly differ in time spent in open (G) or closed arms (H; $n = 11$; t-test; $p=0.789$ and $p=0.153$ for open and closed arms, respectively). Data is expressed as group means \pm S.E.M..

A. Body weights of 6-month-old mice



B. Brain weights of 6-month-old mice

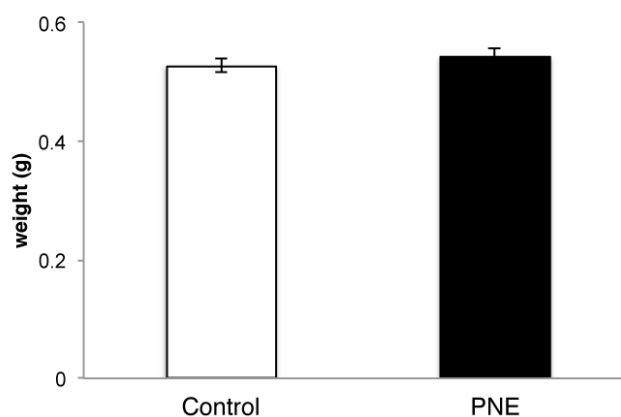
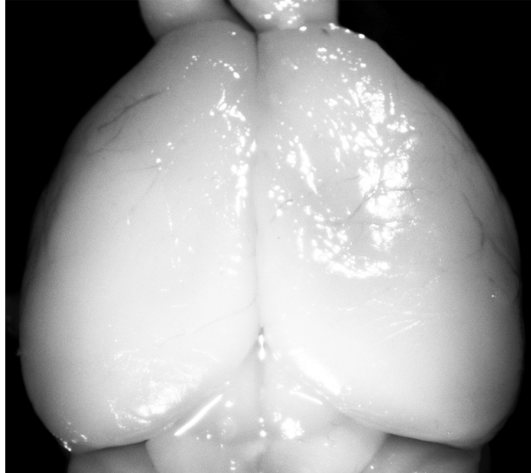


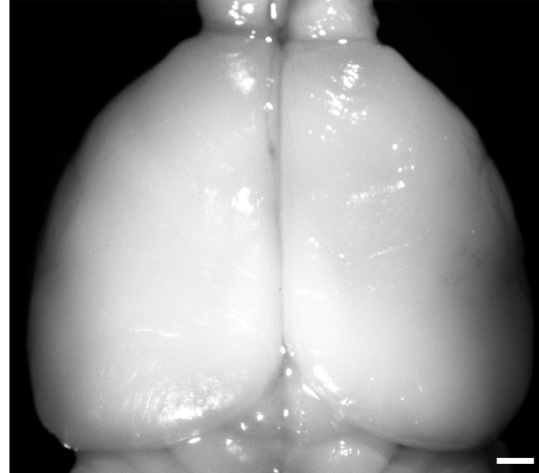
FIGURE 5.3 . *Body and brain weights of 6-month-old control and PNE mice.*

The body weights and brain weights of control and PNE mice were recorded at 6 months of age. No significant differences in body weight were found between control (n=10) and PNE (n=8) mice (A; t-test; $p=0.129$). No significant differences in brain weights between control (n=6) and PNE mice (n=8) were found at six months of age (B; t-test; $p=0.422$). Data is expressed as group means \pm S.E.M..

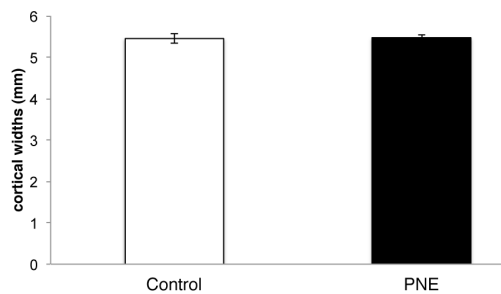
A. 6-month-old control cortex



B. 6-month-old PNE cortex



C. Cortical widths of 6-month-old mice



D. Cortical lengths of 6-month-old mice

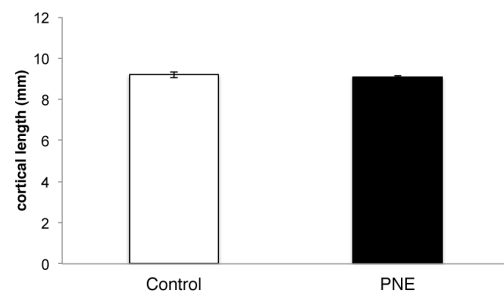


FIGURE 5.4. Cortical size of 6-month-old control and PNE mice.

Dorsal images were taken of 6-month-old control (A) and PNE (B) brains after sacrifice and perfusion and appear to be similar in cortical size. Cortical widths (C) and lengths (D) of control (n=5) and PNE (n=8) brain hemispheres were measured and no significant differences were found (t-test; $p=0.915$ and $p=0.601$ for width and length, respectively). Scale = 2 mm; Rostral is up and lateral to the left and right; Data is expressed as group means \pm S.E.M..

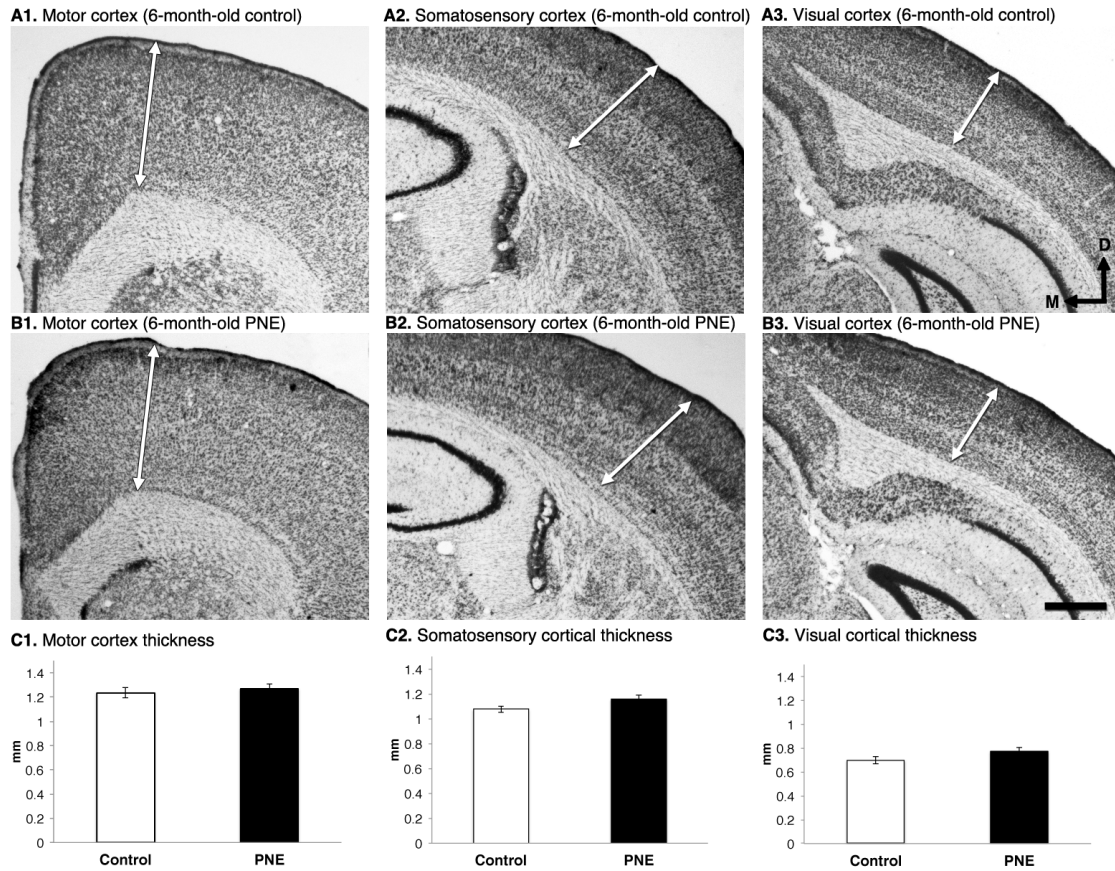


FIGURE 5.5. Cortical thickness of sensory and motor areas in 6-month-old control and PNE mice

Representative nissl-stained sections are shown for control (A1-A3) and PNE (B1-B3) brains. Measurements of cortical thicknesses were measured in motor (A1, B1), somatosensory (A2, B2), and visual (A3, B3) cortices. Double-headed arrows indicate location of measurement. Measurements revealed no significant differences in cortical thickness of motor cortex (C1; $n=3$; t -test, $p=0.604$), somatosensory cortex (C2; $n=3$; t -test, $p=0.104$), or visual cortex (C3; $n=3$; t -test, $p=0.158$). Scale = 1 mm; Dorsal is up and medial to the right; Data is expressed as group means \pm S.E.M.

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