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Neurobiological Effects of Prenatal Ethanol Exposure and Perceived Safety of Ethanol Consumption During Pregnancy

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Neurobiological Effects of Prenatal Ethanol Exposure and Perceived Safety of
Ethanol Consumption During Pregnancy

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

Doctor of Philosophy

in

Psychology

by

David John Rohac

June 2018

Dissertation committee
        Dr. Kelly Huffman, Chairperson
        Dr. Peter Hickmott
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ABSTRACT OF THE DISSERTATION

Neurobiological Effects of Prenatal Ethanol Exposure and Perceived Safety of
Ethanol Consumption During Pregnancy

by

David John Rohac

Doctor of Philosophy, Graduate Program in Psychology
University of California Riverside, June 2018
Dr. Kelly J Huffman, Chairperson

Alcohol exposure during fetal development is the leading known
preventable cause of mental retardation in the western world (Abel et al.,
1995; Abel et al., 1987; Stratton et al., 1996). Animal models and human
studies reveal deficits in sensory-processing, behavior, motor learning, spatial
functioning, and increased anxiety as a result of prenatal ethanol exposure
(PrEE; Glavas et al., 2001; Kalberg et al., 2006; Hellemans et al., 2010; Carr
et al., 2010; Hamilton et al., 2010). Work in the Huffman Laboratory has
demonstrated how PrEE can induce abnormal neocortical gene expression,
miss targeting of intraneocortical connections, changes to brain anatomy, and
altered behavior in the first filial (F1, directly exposed) generation (El Shawa et
al., 2013), as well as ethanol's ability to induce epigenetic modifications
(Abbott et al., 2017). If PrEE-induced epigenetic modifications contribute to the
observed abnormal gene expression in PrEE animals, then it is plausible that
PrEE related conditions could be heritable across generations without further

exposure. By extending the metrics used to assess the first generation, we discovered stable modifications to brain anatomy, connectivity and behavior that is detectable across three generations. Many of the anatomical and connectivity related phenotypes are detectable at birth, and behavior is altered across all three generations when measured in prepubescent mice. Cataloging the effects that persist across multiple generations provides strong evidence for ethanol-induced epigenetic changes during development and provides insights into some of the mechanisms influencing heritability. The final study of this dissertation assesses the general perception of safe dietary behavior during pregnancy including alcohol. Responses demonstrated a lack of understanding about the danger of items containing teratogens like BPA, mercury and prescription pain medications and revealed a misconception about the safety of periodic wine drinking throughout pregnancy. The safety of alcohol as scored on our survey approximated CDC statistics of actual consumption during pregnancy. It is clear that increased education about the dangers of teratogen consumption, such as alcohol, during pregnancy is critical for the health and well being of future generations

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General introduction

Fetal Alcohol Spectrum Disorders

In late 2015, the American Academy of Pediatrics released a statement that there is "no known safe amount of alcohol for a pregnant women to drink". Despite this warning and that scientific research has illuminated the dangers of alcohol consumption during pregnancy, a recent CDC report suggests that 3 in 4 women who want to get pregnant as soon as possible do not stop drinking alcohol when they stop using birth control (CDC 2016). The consumption of alcohol during pregnancy increases the risk of complications in pregnancy and large amounts can lead to complete loss of the fetus (Aliyu et al., 2008; Lundsberg et al., 1997; Maconochie et al., 2007; Strandberg-Larsen et al., 2008; Windham et al., 2015). Alcohol use during pregnancy is still the leading known preventable cause of mental retardation and birth defects in the western world (Abel et al., 1987; Sampson et. al., 1997).

Several organizations, including The American Medical Association, Centers for Disease Control, the Food and Drug Administration, and the American Academy of Pediatrics have made considerable efforts to educate the public on the dangers of alcohol consumption during pregnancy; however, the prevalence of misinformation may contribute to the persistence of Fetal Alcohol Spectrum Disorders (FASD). FASD includes several diagnostic classifications used to identify the severity of the impairment, including partial FAS (pFAS),

Alcohol-Related Neurodevelopmental Disorder (ARND) and Fetal Alcohol

Syndrome (FAS) (Chudley et al., 2005). These range in severity from least to

worst, respectively, and represent a set of recognizable symptoms with

abnormalities in three distinct areas; prenatal and/or postnatal growth deficiency,

central nervous system (CNS) dysfunction and a distinctive pattern of facial

malformations, including a thin vermillion border, smooth philtrum and short

palpebral fissure length (Riley, 2005). The degree to which each individual

displays these characteristics depends on a wide range of variables; animal

model studies have concluded a number of factors can influence the

consequences, including when exposure occurred, the amount consumed,

genetic predispositions, and the nutritional status of the mother (Riley, 2005; May

et al., 1983; Jacobson et al., 1996; May and Gossage, 2011).

Despite recommendations by government agencies to help prevent FASD,

an estimated 1 per 100 (1%) live births show phenotypes of alcohol-related

disorders in the United States (May and Gossage, 2001). Additionally, due to the

difficulties in diagnosing FASD, this number is likely underestimated. More

recently, the CDC found that 6 to 9 out of 1,000 children are born with FAS

(CDC, 2014). Unfortunately, only children with the facial phenotype, growth

malformations and central nervous system dysfunction are diagnosed with FAS,

while children with prenatal alcohol exposure who do not fit all the categories

required for diagnosis may go unreported and untreated. These children have

been found to have significant differences in brain structure including reductions

in the mean size of the brain, frontal lobe, caudate, putamen, hippocampus, cerebellar vermis, and corpus callosum (Astley et al. 2009; Sowell et al. 2001a, b, 2002a, b, 2008b), and they can experience debilitating cognitive and behavioral impairments including deficits in language, motor coordination, learning, memory impairment, and visuospatial functioning (Mattson et al., 1998).

Ethanol alters neuronal function

PrEE may affect neural function and development through a wide range of effects on specific neural cells. PrEE affects neurotrophic factor-mediated pathways (Breese et al., 1995; Maier et al., 1999), retinoic acid synthesis and retinoic acid-dependent signaling pathways (Deltour et al., 1996), cell adhesion molecules (Bearer et al., 1999), and protein kinases (Perrone-Bizzozero et al., 1998). There is also evidence that ion channels are affected by fetal or neonatal ethanol exposure, which may have direct effects on the development of the nervous system. Ethanol has been reported to modulate the $GABA_A$ receptor complex and potentiate the effects of GABA in some preparations (Mihic and Harris 1996). It has also been shown to alter NMDA receptor function. Acute treatment of ethanol inhibits NMDA-induced responses (Hoffman et al., 1989), while chronic intake is reported to increase the number of NMDA receptors in the rat brain.

Ethanol exposure during synaptogenesis induces apoptotic degeneration within the nervous system that removes large numbers of neurons from the parietal and cingulate cortex by blocking NMDA receptors and excessively activating $GABA_A$ receptors (Ikonomidou et al., 2000). The GABAergic system has received a great deal of attention for its role in alcohol behavior and responses. A number of studies have identified mechanisms in the GABAergic system as a key target of ethanol action in the brain that may contribute to the long-term effects of ethanol on the developing brain, including a decrease in the number of GABAergic neurons in layers 2 and 3 of the neocortex, and altered migration of GABAergic interneurons in the cortex (Weiner and Valenzuela, 2006). Neuronal loss in the cortex, coupled with reports of specific loss of cerebellar and hippocampal neurons in the brain as a result of ethanol exposure during development, could be the mechanisms underlying a change in brain volume of children diagnosed with FASD (Ikonomidou et al., 2000; West et al., 1984; Bauer-Moffett and Altman, 1977; Goodlett et al., 1990). Ethanol exposure during gestation delays the migration of cortical neurons. In a study by Miller, cortical neurons were shown to migrate to their final position much slower by remaining within the proliferative zone for longer and by having a slower rate of travel. Specifically, layer 5 neurons in normal developing rat cortex are born on gestation day (GD) 15 to GD17, whereas layer 5 neurons in ethanol-treated rats are born on GD16 to GD18, with ethanol delaying the migration of cortical neurons by up to 2 days. Late-generated neurons appear to be heavily effected

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by ethanol. In normal rats, these neurons migrate to layers 2 and 3; however, in the ethanol treated animals most of the late-generated neurons terminated their migrations in layers 5 and 6 (Miller, 1993). This suggests that alcohol exposure during synaptogenesis may be the underlying cause for the delayed sensorimotor development and impaired fine/gross motor control observed in children with FASD (Burd et al., 2003; Connor et al., 2006; Lopez-Tejero et al., 1986; Streissguth et al., 1984; El Shawa et al., 2013).

Ethanol's effects on brain development

Prenatal ethanol exposure affects all stages of brain development, including gross changes to anatomy, dendritic morphology, disrupted cell-cell interactions, altered gene expression, oxidative stress, and disruptions in growth factor signaling (Goodlett et al., 2005; El Shawa et al., 2014; Abbott et al., 2016). The neocortex appears to be particularly susceptible to insult from prenatal ethanol exposure. Studies have attempted to reveal the pathways by which cortical dysfunction can occur due to PrEE, including migrational defects (Miller 1993), apoptosis (Ikonomidou et al., 2000), altered gene expression, and changes in cortical thickness/length (Sampson et al., 1994; O'leary-Moore et al., 2010; El Shawa et al., 2013; Abbott et al., 2016).

The neocortex is the brain structure responsible for complex behavior and high level cognitive processing in humans. Normal function depends upon a precise network of connections allowing accurate processing of sensory stimuli

between and within cortical areas. The network on connections between cortical areas is also known as intraneocortical connections (INCs). Abnormal development and maintenance of the neocortex may be the neural substrate underlying multiple developmental disorders (Casanova et al., 2002; Daskalakis et al., 2008; Barrat et al., 2008; Sprons, 2011). The establishment of the cortical areas through development is a process called arealization. The exact mechanisms driving arealization are still not fully understood; however, it is clear that gene expression is related. The protomap hypothesis put forward by Rakic suggest that the developing neocortex is "patterned" early in development, prior to the arrival of sensory input, with differential expression of genes during arealization (Rakic 1988; Donoghue and Rakic 1999; Fukuchi- Shimogori and Grove 2003). For example, neocortical gene expression patterns are unchanged in mutant mice lacking thalamocortical afferents (Miyashita-Lin et al. 1999). Recent studies demonstrate that in the reeler mutant mouse, with a severely disorganized neocortex, thalamocortical afferents still make meaningful synaptic connections with layer IV type ectopic cells within the barrel field area that respond to whisker deflection and show early gene activation after stimulus to the whiskers (Wagener et al., 2016).

Input from the sensory systems has been shown to sculpt final cortical organization and function. The protocortex model postulated by O'Leary opposes the protomap and states that the developing neocortex is a *tabula rasa* that is shaped by input from peripheral sensory receptors. Proper development of the

neocortex does not rely solely on intrinsic properties of the cells within, but requires external input from sensory systems for accurate and functional growth. Specifically, In fact, a complex interplay of both intrinsic signals and extrinsic input from senses generates the mature, functional neocortex (for review see Sur and Rubenstein, 2005). This interplay of signals creates a structure that is malleable and subject to organizational disruption due to incorrect signaling. Following insults from the environment, such as alcohol exposure, developmental pathways may be altered and effect the proper development of the brain. Exposure to environmental toxins during critical periods of early development have been shown to cause overwhelming changes to the neurobiology of a fetus, leading to altered cognitive and behavioral phenotypes. Similarly, PrEE has been shown to disrupt patterns of cortical development, hypothesized to be caused by ethanol's affect on DNA methylation levels that could theoretically lead to a heritable change in gene expression (El Shawa et al., 2013; Abbott et al., 2017).

Heritability and epigenetics

Research suggests that children of alcoholic parents are much more susceptible to addiction and alcoholism themselves (Adler et al., 1983). There is consistent evidence that substance use disorders run in families (Bierut et al., 1998). Adoption, twin, and sibling studies suggest genetic factors in the heritability of abuse (Cloninger et al., 1981). Alcohol is included within these studies and developmental studies suggest that exposure to ethanol at specific

developmental stages may result in changes that could persist across generations. Research investigating ethanol's ability to modify epigenetic pathways, some of which have been shown to be maintained between generations, represents a new and exciting area in neuroscience. Epigenetics is defined as a change in gene activity that is not caused by a change in gene sequence itself (Holliday, 1990). Evidence for the effects of epigenetics on development have been documented for some time. The most influential animal study includes work on the agouti mouse. An epigenetic transposon upstream of the *agouti* gene causes its expression to increase and results in a heritable change in phenotype where mice have an altered coat color (yellow) and are obese (Morgan, 1999). Alcohol is theorized to have multiple effects on epigenetic pathways including: one-carbon metabolism, the primary source of methyl donors in DNA-transmethylation reactions (Halsted et al., 2002), DNA methyl-transferase, DNA methylation (Garro et al., 1991), and histone modifications (Shukla et al.,2008). Assessing the methylation state as a correlate for changes in gene activity in an epigenetic mechanism is now being done throughout the field. A study published in 2012 demonstrated this by documenting that males passed on the hypomethylated state of the proopiomelanocortin gene to his offspring, resulting in its dysfunction (Govorko et al., 2012). Additional research has suggested that DNA methylation is altered in embryos exposed to ethanol *in utero* by noting reductions in methylase activity relative to controls even in the presence of excess S-adenosylmethionine, which serves as the methyl donor for

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the enzyme DNA methyltransferase, or altered methylation profiles in embryos with PrEE (Liu et al., 2010). Animals in these studies show severe alterations to development including delayed growth, alterations to the heart, neural tube defects, vesicles, optic system damage and limb bud changes. Additional work by our lab has documented altered methylation of regulatory genes and DNA methyl transferases throughout the brain due to prenatal ethanol exposure (Abbott et al., 2017).

Perceptions of safe dietary habits during pregnancy

Recent work by the Huffman laboratory is exploring ways, such as preventative treatments, to ameliorate the effects of PrEE. Treatment in humans is complicated by the fact that identification of children born with FASD requires a maternal admission of alcohol consumption, something not always easy to obtain. At the moment, it is apparent that the most effective method of reducing the prevalence of FASD is through prevention. In order to prevent pregnant women from exposing their unborn offspring to ethanol, we must educate the public about the dangers of drinking in pregnancy. The study in Chapter 3 aims to understand what basic food, beverage and medication products people perceive as safe or unsafe so that we can identify specific loci and groups where education is lacking.

Fetal outcomes are linked to the mother's dietary consumption habits during pregnancy (Kaiser et al., 2010; Keen et al., 2003). The general public may

not have access to or be aware of the most recent scientific conclusions regarding dietary behavior and pregnancy; thus, it is important that we understand the current trends in perception. Beliefs about the safety of specific foods and drinks during pregnancy could strongly influence their decisions regarding dietary behavior during pregnancy. This may be true for pregnant women, or their partners who may influence decisions. It is therefore important that we understand the perception of both men and women when it comes to safe pregnant dietary behavior.

Importantly, reports suggest that the use of prescription pain medications, namely opiates, has been increasing over the last several decades. A recent study found that between 28% and 39% of women at reproductive age (15-44) filled a prescription for an opioid medication, with differences based on insurance (CDC 2015). It is important that we continue to push research into the implications of this trend and understanding the perceived safety of opiates may be the first step in guiding education to correct this.

Although primarily focused on alcohol, the study in chapter 3 also includes other teratogens, some lesser known, with implications for fetal development. A study published in our lab demonstrated that, despite the work done in science to discover the importance of healthy dietary choices during pregnancy, a proportion of pregnant women consume substances that are potentially harmful to their baby (Santiago et al., 2013). Understanding the gaps in public perception

versus science is the first step in developing education towards correcting the misinformation.

The first two chapters of the dissertation extend our initial findings on the effects of prenatal ethanol exposure to a transgenerational model that tracks heritable effects on brain development and behavior. We also investigate whether aberrant INCs, and altered brain anatomy documented in newborn mice persist into older ages. The study described in the third chapter seeks to explore the perceptions that people have about safe dietary practices during pregnancy. We assess not only the perception of alcohol's safety during pregnancy, but extend to include common products or chemicals such as BPA in canned food, methyl mercury in fish, caffeine in coffee/soft drinks and the safety of medication use during pregnancy. Results from this dissertation provide novel insight into the heritable, epigenetic effects of prenatal ethanol exposure, how it persists through early development and provides insight into the public's current awareness of safe dietary behavior during pregnancy.

Chapter 1: Neuroanatomical effects of prenatal ethanol exposure in newborn (P0) mice: a transgenerational model

Introduction

Prenatal exposure to alcohol, or ethanol (PrEE), disrupts brain and behavioral development in humans leading to Fetal Alcohol Spectrum Disorders (FASD). FASD describes a range of phenotypes in offspring whose mothers consumed

alcohol during pregnancy (Lemoine et a., 1968; Jones et al., 1973). Results from multiple studies have demonstrated that PrEE generates deficits in sensory-processing, behavior, motor learning, spatial functioning, and anxiety (Kalberg et al., 2006; Hellemans et al., 2010). Previous research from our laboratory in PrEE has demonstrated a host of phenotypes in the exposed offspring (first filial generation, F1). Specifically, we have shown changes in neocortical gene expression, development of ectopic connections between two sensory areas, neuroanatomy and abnormal behavior due to *in utero* ethanol exposure (El Shawa et al., 2013; Abbott et al., 2016). PrEE induced neocortical disorganization and altered brain anatomy may represent underlying substrates for sensorimotor, perceptual, cognitive, and behavioral deficits observed in humans with FASD.

Alcohol consumption during pregnancy increases the risk of complications and pregnancy loss (Aliyu et al., 2008; Strandberg-Larsen et al., 2008; Windham et al., 2015). Despite the conclusions of the majority of scientists in the field that there is "no safe amount" of alcohol that can be consumed by a mother during pregnancy, the CDC found that between 0.2 and 1.5 infants are born with FAS for every 1,000 live births (CDC, 2006-2010). The most recent CDC statistics suggest "an estimated 3.3 million women between the ages of 15 and 44 years are at risk of exposing their developing baby to alcohol because they are drinking" (CDC 2016).

Significant attention has been focused on children directly exposed *in utero* to alcohol; however, the more we explore the epigenetic pathways involved in inheritance the more we discover how deleterious traits may be passed on. Couple this with studies suggesting the children of alcoholics are more susceptible to alcohol addiction themselves (Adler et al., 1983) and these patterns of inheritance begin to emerge. This idea has recently been corroborated in animal studies suggesting that PrEE increases alcohol consumption and sensitivity in later generations (Nizhnikov et al., 2016). A prevailing question that has emerged from our laboratory's data is: could the phenotypes in the F1 offspring be present in subsequent generations? Evidence is mounting for ethanol's ability to modify epigenetic pathways, subsequently resulting in a heritable pathology. Recently published work in our lab has demonstrated that PrEE induces epigenetic modifications in mice (Abbott et al., 2017). In that study numerous changes were found including the up regulation of neocortical gene expression along with promoter hypomethylation of specific genes (*Rzr*β & *Id2*) as well as decreased global DNA methylation. DNA methyl transferase (DNMT) expression was also lower in newborn cortex. We have hypothesized that ethanol-induced changes to gene expression in the exposed offspring, via epigenetic modification, contributes to the anatomical and behavioral phenotypes in F1 PrEE mice. The primary question is this: are the changes to anatomy and behavior caused by PrEE stable and do they persist through reproduction?

Due to germ cells exposure within the first generations embryo, any effects seen in the second generation would be considered intergenerational transmission (Sarkar, 2015). Effects that persist to the third generation would represent transgenerational epigenetic modification (Gapp et al., 2017). This distinction is important and dictates why we explored the effects beyond the second generation. We hypothesized that moderate PrEE in the mouse would result in alterations in region-specific cortical thickness that would persist from the first into the third generation, where direct exposure is no longer the cause. Despite the importance of deep gray matter structures such as the basal ganglia, hippocampus, amygdala, and thalamus for motor function, cognition, memory, and emotional networks (Mendoza and Foundas, 2008), there is a paucity of research exploring changes to these areas as a result of PrEE. It is because of this we targeted not just primary sensory and structural components of the cortex, but we included several subcortical structures as well in order to catalogue their sensitivity to PrEE insult.

Materials and methods

All breeding and experimental studies were administered after careful consideration of the protocol guidelines approved by the Institutional Animal Care and Use Committee at the University of California, Riverside. The CD1 Background mice used for breeding in the experiment were purchased from Charles River Laboratories. Mice are housed in an environmentally controlled

environment kept at approximately 22 degrees Celsius and are kept on a 12 hour light/dark cycle. Mouse chow and water are provided *ad libitum*.

Ethanol administration

Beginning on day G 0.5 females in the experimental group are given 25% ethanol solution in water until birth. Both the ethanol solution and mouse chow were provided *ad libitum*. Water and mouse chow were provided to the control dams *ad libitum*. This exposure paradigm is not designed to mimic any specific drinking habits observed in humans; however, it does produce an average blood alcohol content of around 100-140 mg/dl at peak times of consumption (El Shawa et al., 2013). Although this is high in human standards, murine models demonstrate a greater ability to break down alcohol based on a much higher metabolic rate, especially in mice (Cederbaum, 2012) and relates to blood alcohol contents within range of human consumption.

Food and fluid consumption

Food and fluid consumption were measured each day to assess potential confounding differences between F2, F3 and control dams in terms of caloric intake. The weight of the mouse chow was measured using a standard Fisher Scientific Scale. Liquid intake of water and ethanol solution was measured using a graduated drinking bottle. Throughout the gestation process, each dam's body weight was measured using a standard Fisher Scientific scale daily. The final

weight was determined the day before birth. This was used to determine the total weight gained by the dams.

Breeding of first filial generation mice

The first filial generation of mice exposed to ethanol prenatally (F1 PrEE) was bred using a maternal model of self-administration of 25% ethanol (El Shawa et al., 2013). Two control ninety-day-old female mice, who had not given birth previously, were paired with one control male of the same age in the evening, just before their dark cycle. Conception is signified by the observation of a vaginal plug. On the day of conception, Gestational day (G) 0.5, pregnant females are moved to a separate cage. Dams were weight-matched, separated into ethanol-treated and control groups, and provided *ad libitum* access to standard mouse chow.

Breeding of second and third filial generation mice

F1 PrEE male offspring generated from female mice that consumed 25% ethanol throughout gestation were used to breed the F2 generation. All mice were weighed on the day of birth and were inspected for any obvious developmental anomalies. PrEE F1 pups were cross-fostered with a control dam. Pairing F1 males with control females produced the F2 generation. Females were separated from the male on the day a vaginal plug was observed. Dams generating the F2 generation were fed standard mouse chow and water *ad libitum*. Breeding of the

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F3 generation was done in the same way as breeding of the F2 generation. A summary of the breeding paradigm can be seen in Figure 1. Food and fluid intake were monitored to assess any confounding changes in caloric and fluid intake as compared to control type pregnant dams. Weight gain of the dams was also recorded daily until the day prior to birth.

Tissue preparation

On the day of birth (P0), control, F1, F2, and F3 litter size was recorded and each pup was weighed. Pups to be used for P0 Nissl stain analyses were euthanized with a lethal dose of sodium pentobarbital (100 MGN/kg) and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer, pH 7.4. The brains were dissected from the skull, weighed, hemisected, and postfixed in 4% PFA for 6 hours.

Anatomical Tracing

To observe INC development in newborn F1 mice (directly exposed to ethanol *in utero* via maternal consumption), F2 mice (descendant from PrEE F1 males) and F3 mice (descendant from PrEE F2 males), crystals of 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI; Invitrogen) and 4-(4-(dihexadecylamino)styryl)-N-methylpyridinium iodide (DiA; Invitrogen) were placed in the developing neocortex. Single dye crystals were placed into two sensory areas: putative somatosensory cortex (SomCx) or putative visual cortex (VisCx). The crystals

were inserted into the cortex perpendicular to the surface to a depth where the tip of the crystal was level with the cortical surface. After dye placement, the brains were stored in 4% PFA, at room temperature, for a period of 4–6 weeks to allow for transport of tracer. Labeled cells observed in the dorsal thalamic nuclei were used as indices for adequate transport of the tracer. Brain hemispheres were embedded in low-melting point agarose and sectioned in the coronal plane into 100 um-thick sections using a vibratome. Sections were counterstained with 4', 6-diamidine-2- phenylindole dihydrochloride crystallized (DAPI; Roche), mounted onto glass slides, coverslipped with Vectashield mounting medium for fluorescence (Vector Laboratories), and viewed under multidimensional imaging. The location of the dye placement was verified for each case using thalamocortical labeling observed in sections of experimental and control brain hemisections. As had been done previously (Huffman et al., 2004) putative somatosensory cortex dye placement locations (DPLs) were included in our analysis if retrogradely labeled cells were observed in the ventral posterior (VP) nucleus of the dorsal thalamus. Putative visual cortex DPLs were included in our analysis if retrogradely labeled cells were observed in the dorsal lateral geniculate nucleus (dLGN).

INC analyses

All experimental and control sections were imaged with three different filters using a Zeiss Axio Imager Upright Microscope equipped with fluorescence, and

captured using a digital high-resolution Zeiss Axio camera connected to a PC

running Axiovision software (version 4.7). The filters used were as follows: blue

for DAPI counterstain, red for DiI, and green for DiA labeling (excitation

wavelengths: blue, DAPI 359 nm; red, Cy 3 550 nm; green, GFP 470 nm;

emission wavelengths: blue, DAPI 461 nm; red, Cy 3 570 nm; green, GFP 509

nm). The three images were merged and saved in high resolution TIF format.

The amount of dye spread, also referred to as projection zone, was determined

by measuring the extent of retrogradely labeled cells from the DPL as a

percentage of cortical length by counting sections within which dye was detected.

The size of the injection site was also determined as a percent of total cortical

length by counting dye spread across sections. Fluorescence microscopic

images of control and experimental F1-F3 P0 brains labeled with dye in the

putative VisCx and SomCx were used to calculate estimated number of labeled

cells.

Anatomical measures

Brain weight and cortical length were recorded post mortem after dissection from

the skull and prior to sectioning for neuroanatomical measurements. Images to

be used for cortical measures were taken from Nissl stained sections. Sections

were measured across all cases using an electronic micrometer in Image J.

Thickness was determined using a line perpendicular to the cortical surface

extending from layer 1 to the base on layer 6 just above white matter. Cortical

thickness measures included the dorsal frontal cortex, prelimbic cortex, the somatosensory cortex, auditory cortex, and visual cortex. Cortical areas were identified with a developing mouse brain atlas (Paxinos 2007) using anatomical landmarks such as the genu of the corpus callosum and the fimbria of hippocampus. Sub-cortical structure measures included the corpus callosum, CA3 sub region of the hippocampus, dorsal lateral geniculate nucleus, medial geniculate nucleus, ventral-posterior nucleus, basal ganglia and the amygdala. Corpus callosum thickness was measured from the midline region. Hippocampal measures were taken from the CA3 subfield by measuring the thickness of the pyramidal cell layer. Boundaries for dorsal lateral geniculate nucleus (dLGN) were based on the intergeniculate leaf for the ventral lateral border, the external medullary lamina for the ventral medial border, the lateral posterior nucleus for the dorsal border, and the brachium of the superior colliculus for the dorsal lateral border. Boundaries for medial geniculate nucleus (MGN) were based on the superior thalamic radiation as the medial border, the intergeniculate leaf as the lateral border, and the zona incerta as the ventral border. Ventral posterior (VP) measures were taken using the medial lemniscus as the ventral border, the external medullary lamina as the dorsal border, and the ventral posterior-lateral nucleus as the lateral border. Measures of basal ganglia included the caudate putamen, globus pallidus, and ventral palladium. Amygdala measures included the central amygdala, basolateral, and basomedial nuclei. Basal Ganglia, amygdala, VP, MGN and dLGN volumes were calculated by drawing borders

around the specific structure in serial sections at a fixed magnification using ImageJ and multiplying the area measured by the thickness of the combined sections to generate an estimated volume metric. Brains post-fixed in 4% PFA were cryoprotected in 30% sucrose overnight then cut into 40 um thick sections on a cryostat. Sections were then stained following the standard protocol for Nissl staining, cover-slipped with Permount and imaged using a Zeiss Axio camera connected to a Zeiss Discovery.V12 stereomicroscope.

Statistical Analyses

Results are presented as mean ± S.E.M. ANOVA with Tukey's *post hoc* analysis was used to establish significant differences between dam measures (weight grain, food consumption, liquid consumption, and litter size), pup measures (body weight, brain weight, cortical thickness, neuroanatomical volume, cell packing density), and dye tracing analysis of F1, F2, F3 and control mice. For data displayed as percent change, mean baseline corrected control was set as 100%, with experimental measures expressed as percentage variation from control mean. For dye tracing quantification, dye spread was measured and reported as percent of cortical length by counting the extent to which the dye placement could be identified across 100-um thick sections. Projection zones for each dye were determined by measuring the extent of retrogradely labeled cells from the placement location as a percentage of cortical length. Additionally, cell counts within areas of ectopically labeled cells were calculated and compared across

conditions using ANOVA with Tukey's *post hoc* analysis to highlight the difference in labeling. An odds ratio with confidence intervals of rostral/caudal labeling from putative VisCx and SomCx was calculated to show the variability among cases.

Results

Dam Measures

Several metrics were recorded to track the gestational period and look for confounds caused by changes in consumption during the pregnancy. To ensure our exposure paradigm did not result in malnutrition or dehydration, measures of food intake, liquid intake, dam weight and blood plasma osmolality were recorded. No significant differences in food intake between control and experimental animals (Figure 2A; control, 6.928 ± 0.314 g/day; F1, 6.987 ± 0.532 g/day, p=0.999; F2, 7.328 ± 0.401 g/day, p=0.914; F3, 7.244 ± 0.434 g/day, p=0.965). Gestational weight gain was significantly reduced in F1–F3 animals when compared to controls (Figure 2B; control, 24.44 ± 1.381 g; F1, 19.70 ± 0.770g, p<0.05; F2, 19.03 ± 0.894g, p<0.05; F3, 18.93 ± 2.153 g, p<0.05); a reduction in litter size in all 3 experimental generations was also observed (Figure 2C; control, 11.250 ± 0.470; F1, 9.043 ± 0.571, p<0.05; F2, 8.750 ± 0.636, p<0.05; F3, 8.250 ± 0.560, p<0.05). Dam plasma osmolality showed no significant differences between groups (Figure 2D; control, 308.2 ± 1.773 mosm/kg; F1, 309.2 ± −1.645 mosm/kg, p=0.977; F2, 310.3 ± 2.161 mosm/kg,

p=0.871; F3, 309.5 ± 2.237 mosm/kg, p=0.965). Dam BEC measures revealed

blood ethanol only in F1 dams, with an average of 104.4 ± 1.206 mg EtOH/dl at

GD9, increasing to 135.2 ± 4.126 mg EtOH/dl at GD19, with no ethanol detected

in F2 and F3 dams (Abbott and Rohac et al., 2017).

Pup measures

For this study we measured body weights, brain weight and cortical length in

newborn (P0) F1, F2, F3 and control mice. The brains of F1, F2 and F3 mice

were smaller as compared to controls on the day of birth (Figure 3 & 4). On the

day of birth (P0) F1, F2 and F3 mice had significantly lower body weight (control=

1.69 ± 0.052g; F1= 1.37 ± 0.0633g, p<0.001; F2= 1.38 ± 0.050g, *p<0.001*; F3=

1.49 ± 0.055g, *p<0.01*) (Figure 3A) and brain weights (control= 0.09986 ±

0.0038g; F1= 0.08714 ± 0.0035g, p< 0.01; F2= 0.08972 ± 0.0019g, *p<0.01*; F3=

0.08974 ± 0.0018g, *p<0.05*) (Figure 3b) along with reduced cortical length

(control= *4.458* ± 0.080g*;* F1= 4.015 ± 0.0733g, p<0.001 F2= 4.041 ± 0.0360 g*,*

p<0.001; F3= *4.227* ± 0.0558 g, *p<0.05*) as compared to controls (Figure 4).

Intraneocortical connections

Study of the ipsilateral INCs in the F1–F3 P0 mouse cortex demonstrated that

the observed aberrant connectivity in PrEE F1 mice persisted to the unexposed

F3 generation (Figure 4, Abbott et al., 2017). Labeled cells from putative

somatosensory cortex DPLs (Figure 5, A2–D2, stars) were detected caudally

within cortex of F1–F3 animals (Figure 5, B4–D4, arrows), where no labeled cells were observed in control animals (Figure 5, A4). Dye labeling resulting from putative visual cortex DPLs (Figure 5, A5–D5, stars) revealed an extended region of aberrant connectivity in F1–F3 mice which projects to far rostral regions of cortex. Axonal projections from these labeled cell bodies extend from an area within the frontal, motor cortex (Figure 5), B1–D1, B2–B3, C2–C3, D2–D3) to visual cortex; a pattern not present in the cortex of age-matched control mice (Dye et al. 2011a, 2011b).

Despite having similar DPL size (Figure 6 A & D, Abbott et al., 2017), analysis of DPL and projection zones across the cortex demonstrated that experimental animals had significantly larger projections in somatosensory cortex of F1 and F2 animals, with near significant increases in F3 (Figure 6B; control, $39.4 \pm 2.57\%$; F1, $62.8 \pm 13.0\%$, $p<0.001$; F2, $56.3 \pm 1.28\%$, $p<0.05$; F3, $53.1 \pm 3.44\%$, $p=0.07$). Visual cortex projection zones were also expanded in all experimental generations when compared to controls (Figure 6E; control, $45.6 \pm 2.12\%$; F1, $79.1 \pm 1.9\%$, $p<0.0001$; F2, $60.5 \pm 2.00\%$, $p< 0.001$; F3, $56.5 \pm 3.39\%$, $p<0.05$).

The PrEE-induced INC disruption varied, with some animals showing reduced or absent labeling in far rostral or caudal locations. The odds ratio of F1–F3 animals presenting ectopic rostral labeling was significant across all generations ($p<0.01$), with a high percentage of cases displaying the phenotype (F1, 75.00%; F2 72.73%; F3, 71.43%). The odds ratio of F1–F3 animals

presenting ectopic caudal labeling faded across generations and significance

was detected into the F2 generation, with F3 mice approaching significance (F1,

p<0.01; F2, p<0.05; F3, p=0.054). To control for variability, cell counts were

performed only in cases expressing the phenotype (control n=10, F1 n=9, F2

n=10, F3=10). Cell counts within rostral cortical areas revealed significant

numbers of ectopically labeled cells arising from a visual cortex DPL, a

phenotype that persisted into the F3 generation [F(3,34) = 6.022, p<0.001]. Post

hoc analyses indicated significant variance in labeled cells in all 3 experimental

groups, when compared to controls (Figure. 6F; control, 0.0 ± 0.0; F1, 55.5 ±

12.64, p<0.01; F2, 43.0 ± 12.87, p<0 0.05; F3, 51.6 ± 12.1, p<0.01). Cell counts

within these caudal regions of cortex revealed a similarly significant increase

[F(3,20) = 13.76, p<0.0001] of labeled cells in F1, F2, and F3 generations when

compared to controls (Figure 6C; control, 0.0 ± 0.0; F1, 225.2 ± 20.79, p<0.0001;

F2, 199.8 ± 41.46, p<0.001; F3, 192.2 ± 31.33, p<0.001).

Anatomical measures

Analyses of anatomical measures revealed additional changes in F1, F2 and F3

mice as compared to controls on the day of birth. Volumetric and thickness

measures taken from Nissl stained sections at P0 revealed that F1, F2 and F3

mice displayed significant differences in select brain regions.

Frontal cortex: Significant thickening of dorsal frontal cortex due to PrEE was seen in experimental mice (Figure 7 A1-A4; control 100 ± 2.9%, F1 116.7 ± 6.02%, F2 120.1 ± 4.45%, F3 119.7 ± 3.89%) [F(3, 65) = 4.673, *p=0.0051*]. Tukey's post hoc analysis revealed the differences between F1, F2 and F3 when compared to controls were significant at *p<0.05* (Figure 7 A5).

Prelimbic cortex: Analysis of the prelimbic cortex revealed a thinning of cortex that persisted across generations (Figure 7 B1-4; control 100 ± 2.04%, F1 84.61 ± 4.46%, F2 88.28 ± 2.42%, F3 88.73 ± 2.93%) [F(3,50) = 6.724, *p=0.0007*]. Post hoc analysis revealed significant differences compared to controls (F1, *p<0.01; F2 & F3 p<0.05*) (Figure 7 B5).

Somatosensory cortex: Analysis of the somatosensory cortex revealed a thickening of cortex that persisted across generations (Figure 7 C1-C4; control 100 ± 2.09%, F1 118.4 ± 5.83%, F2 115.9 ± 3.17%, F3 116.3 ± 4.21%) [F(3,34) = 4.732, *p=0.0073*]. Post hoc analysis revealed significant differences in all three generations compared to controls (F1, F2 & F3 *p<0.05*) (Figure 7 C5).

Auditory cortex: Measurements taken from the primary auditory cortex also showed a significant thinning of experimental auditory cortex compared to control cases (Figure 6 D1-D4; control 100 ± 2.34%, F1 84.19 ± 2.71%, F2 101.4 ± 1.89%, F3 98.99 ± 2.82%) [F(3,34) = 12.38, *p<0.0001*]. Post hoc analysis

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showed a significant thickening of the F1 generation (*p<0.001) (*Figure 7 D2)*, but no differences in F2 and F3 (Figure 7 D5).

Visual cortex: Additional measures from visual cortex revealed a significant thickening of cortex (Figure 7 E1-E4; control 100 ± 3.67%, F1 114.7 ± 4.04%, F2 96.78 ± 2.56%, F3 95.82 ± 3.22%) [F(3,36) = 6.599, *p=0.0011*]. Post hoc analysis revealed a significant thickening in the F1 generation mice (*p< 0.05*) (Figure 7 E2), but not the F2 and F3 generation (Figure 7 E5).

Corpus callosum: Corpus callosum thickness was reduced due to PrEE (Figure 8 A1-A4; control 100 ± 2.31%, F1 79.73 ± 4.88%, F2 80.46 ± 5.74%, F3 79.97 ± 5.97%) [F(3,42) = 4.728, *p=0.0062*]; post hoc analyses showed the differences were seen in all generations compared to controls (*p<0.05,* Figure 8 A5).

Hippocampus: Thickness measures of the CA3 region of the hippocampus revealed significant thinning of experimental cases compared to controls (Figure 8 B1-B5; control 100 ± 1.32%, F1 88.15 ± 3.37%, F2 103.9 ± 3.6%, F3 107.4 ± 3.03%) [F(3, 39) = 9.379, *p< 0.0001*], with post hoc analysis revealing differences between F1 and control cases (Figure 8 B1-B2; p=0.0389), but not compared to F2 or F3 animals (Figure 8 B5; F2 p=0.7992, F3 p=0.3143).

Dorsal Thalamic nuclei: Measurements targeting the volume of thalamic sensory nuclei revealed no significant changes compared to control cases. Measures taken from the dorsal lateral geniculate nucleus were not different between control and PrEE cases (Figure 8 C1-C4; control $100 \pm 2.83\%$, F1 $93.13 \pm 4.42\%$, F2 $90.28 \pm 1.81\%$, F3 $90.2 \pm 3.04\%$) [$F_{(3,38)} = 2.114$, $p=0.1145$]. Measurements taken from the medial geniculate nucleus were not significantly different (Figure 8 D1-D4; control $100 \pm 4.48\%$, F1 $99.84 \pm 2.61\%$, F2 $99.15 \pm 8.11\%$, F3 $95.54 \pm 10.22\%$) [$F_{(3,16)} = 0.08907$, *p = 0.9650)*. Volumetric measurements of VP of the thalamus showed no difference across groups (Figure 8 E1-E4; control $100 \pm 4.20\%$, F1 $90.14 \pm 3.24\%$, F2 $89.58 \pm 3.68\%$, F3 $92.19 \pm 3.15\%$) [$F_{(3,24)} = 1.796$, $p=0.1748$].

Amygdala: Volumetric measurements from the Amygdala showed a significant reduction in size (Figure 9 A1-A4; control $100 \pm 5.14\%$, F1 $80.95 \pm 3.67\%$, F2 $78.07 \pm 5.72\%$, F3 $78.57 \pm 2.92\%$) [$F_{(3,24)} = 5.413$, $p=0.0055$], with post hoc analyses showing significant group differences between control, F1, F2 and F3 cases ($p<0.05$) (Figure 9 A5).

Basal ganglia: Finally, measures from the basal ganglia also revealed a significant reduction (Figure 9 B1-B4; control $100 \pm 4.11\%$, F1 $87.18 \pm 2.84\%$, F2 $90.38 \pm 3.17\%$, F3 $93.75 \pm 1.74\%$) [$F_{(3,50)} = 3.102$, $p=0.034$], with post hoc analysis revealing F1 PrEE mice had a significantly smaller BG (Figure 9 B1-B2),

but F2 and F3 animals did not show a difference compared to controls (Figure 9 B5).

Discussion

In this study we explored the transgenerational effects of prenatal ethanol exposure by assessing the newborn offspring of prenatally exposed males. Our data demonstrates wide spread changes to neuroanatomy and connectivity that persist through reproduction

Morphological similarities in PrEE F1, F2, and F3 mice.

Despite the fact that nutrition and water consumption was not different in the mice pregnant with F1, F2 and F3 generation, they gave birth to pups that were smaller and whose brains were reduced in cortical length and weight. Data from this PrEE model demonstrates reduced newborn body weight, brain weight, and reduced cortical length suggesting that ethanol can perturb physical and neocortical development across generations. The reduced weight gain by pregnant dams during gestation may be a result of reduced pup weight and litter size. The results showing no differences in food intake across dams suggests the reduced weight gain not due to food intake. The continued presence of these general developmental abnormalities into unexposed F3 animals presents a new

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and troubling outcome of PrEE and supports the hypothesis that body weight can be regulated transgenerationally by epigenetic factors (Waterland et al. 2008).

Connectivity is altered within neocortex

Intraneocortical connections (INCs) provide the network for sensorimotor integration in cortex. Our laboratory has previously described the disruption of this network in directly exposed F1 PrEE mice (El Shawa et al. 2013). Here, we extend the assessment across generations to explore the transgenerational impact of PrEE on sensory INC development on first, second and third generation mice. The disruption seen in F1 animals is persistent and detectable in both the second and third generation. The altered connections and ectopic cells may be contributing to some of the changes in anatomical structure. Ectopically labeled cells in frontal cortex are regionally correlated with reduced cortical thinning (Treit et al. 2014). This change to the intraneocortrical network may be a result of epigenetic modification of the regulatory genes involved with cortical network development. Previous work has demonstrated that PrEE alters the methylation profiles and changes the expression of specific genes within the developing neocortex seen in F1 through F3 animals (El Shawa et al., 2013; Abbott et al., 2017). Proper expression of these genes is strongly correlated with normal development of cortical areas at the age studied here (Dye et al., 2011a).

Neuroanatomical changes across three generations of PrEE mice

The central nervous system anomalies documented in FASD range from cellular and molecular aberrations to gross structural brain abnormalities (Norman et al. 2009; Riley et al. 2004). Among the most consistent findings in brain imaging studies of FASD is the reduction in overall brain volume (Mattson et al. 1998; Archibald et al. 2001; Willoughby et al. 2008; Norman et al. 2009). The reductions in volume are detected throughout the brain, with frontal, temporal, and parietal lobes showing the most significant effects in individuals with FASD as compared with controls (Archibald et al. 2001). Here we show that similar changes in CD-1 mice caused by PrEE in F1 mice are also seen in their offspring. F1, F2 and F3 mice, on the day of birth, had smaller brains compared to age-matched control animals.

Of the most prominent changes in multiple studies are changes to the corpus callosum. Imaging studies include cases of complete agenesis of the corpus callosum (Astley et al., 2009; Swayze et al., 1997), partial agenesis and/or callosum thinning (Autti-Ramo et al., 2002). At P0 all three generations of mice showed a significantly thinner corpus callosum. Subcortical structures were not immune to the PrEE insult; the overall volume of the amygdala was reduced in F1, F2 and F3 animals. This suggests a dramatic change to the development of multiple brain areas that are impacted differentially; with some changes being corrected in the second generations while some alterations persists.

The mechanisms by which these brain areas are changed may be brought

about via two general pathways: reduced neurogenesis or increased cell death.

Both mechanisms have been implicated in previous studies (Dunty et al., 2001;

Green et al., 2007; Guerri et al., 2009; Livy et al., 2008) and both may be altered

due to PrEE. To test for this possibility we also conducted a cell packing density

analysis within the areas targeted for cortical thickness. Measures of cortical cell

density revealed no difference in F1, F2 and F3 brains compared to controls at

P0 (Table 2); suggesting the changes in cortical thickness may not be derived

from changes to cell packing, but are generated through some other mechanism.

Possible mechanisms governing the heritability of FASD phenotype

One possible mechanism by which this heritable phenotype is being passed on is

an alteration in epigenetic pathways. DNA methylation is a known mechanism

that is involved with gene transcription silencing (Moore et al., 2013). Recent

evidence suggests that alcohol exposure alters methylation profiles of mice when

exposed in utero during neurulation (Liu et al., 2010). Additional work published

by our lab has shown PrEE results in alterations in intraneocortical connectivity,

up regulation of neocortical *Rzr*β and *Id2* expression accompanied by promoter

hypomethylation and decreased global DNA methylation levels across

generations. DNMT expression was also suppressed (Abbott et al., 2017).

Combined with additional work from our lab, this data suggests that changes in

DNA methylation may be altering transcription of select developmental genes

pertinent to cortical development, culminating in changes to observed behavior. We know from additional research that cortical patterning is governed by gene expression and that alterations can alter the boundaries of sensory areas (Fukuchi-Shimogori et al., 2001; Huffman et al., 2004).

It has been proposed that an epigenetic basis of transgenerational effects could be considered when the effects persist through three generations in an exposed female parent (Jirtle et al., 2007; Gapp et al., 2017). The effects documented in this study support the theory that maternal consumption of alcohol during pregnancy has the potential to induce stable epigenetic alterations; thus leading to the persistence of the F1 PrEE phenotypes observed across three generations. Finally, the studies from our lab into the epigenetic inheritance of PrEE-induced developmental changes should provide further support for abstaining from alcohol consumption during pregnancy and further research into the damaging effects.

Chapter 2: Abnormal neuroanatomical and behavioral development in pre-pubescent (P20) mice after prenatal ethanol exposure: a transgenerational model.

Introduction

Prenatal ethanol exposure (PrEE) results in maladaptive changes in brain and behavioral development in mice that persist across at least three generations (Abbott et al., 2017). Specifically, we found PrEE-induced changes in

intraneocortical connections, neocortical gene expression, sensori-motor

coordination, and anxiety that persisted to the third filial (F3) generation in

newborn mice. In this chapter, we investigate whether some of the

transgenerational changes observed in PrEE newborn mice persist to a later age,

twenty-day-od (P20) mice. Measurements of various neuroanatomical structures,

analyses of intraneocortical connections (INCs) and behavioral assays were

studied in PrEE F1-F3 and control P20 mice. Behavioral tests included assays

devised to assess motor control, sensori-motor integration, anxiety, depression

and social behavior.

Children with fetal alcohol spectrum disorders (FASD) may have birth

defects, facial abnormalities, health problems, motor delays, language and

learning disabilities, attention deficits, and memory problems. Additionally, due to

the recently discovered transgenerational effects of PrEE, there is no research

tracking behavioral alterations across generations after the initial exposed

generation. Children diagnosed with FASD or those with confirmed prenatal

alcohol exposure can display impairments in social competence (Brown et al.,

2004; Olson et al., 1997; Mattson and Riley, 2000) social relationships (Bishop et

al., 2007; Thomas et al., 1998) and have problems with anxiety, depression and

fine motor skills (Brown et al, 2018). Deficits in social skills caused by FASD

cannot be fully explained by deficits in intellect (Mattson and Riley, 2000) and

typically become more prominent with age (Thomas et al., 1998; Whaley et al.,

2001).

This study seeks to track behavioral disruptions using a transgenerational mouse model along with several key neuroanatomical parameters that may be mediating these behaviors. To accomplish this task, we performed behavioral assessments targeting motor control, sensorimotor integration, social behavior, depression and anxiety while assessing changes in brain anatomy and connectivity of a select set of cortical structures, several thalamic nuclei as well as the anatomy of the hippocampus, the corpus callosum, basal ganglia and the amygdala.

Materials and methods

All breeding and experimental studies were administered with strict adherence the protocol guidelines approved by the Institutional Animal Care and Use Committee at the University of California, Riverside. The CD1 mice used for breeding in the experiment were purchased from Charles River Laboratories. Mice are housed in an environmentally controlled environment kept at approximately 22 degrees Celsius and are kept on a 12 hour light/dark cycle. Mouse chow and water are provided *ad libitum*.

Ethanol administration

Beginning on day G 0.5 females in the experimental group are given 25% ethanol solution in water until day of birth. Both the ethanol solution and mouse chow were provided *ad libitum*. Water and mouse chow were provided to the

control dams *ad libitum*. This exposure paradigm is not designed to mimic any specific drinking habits observed in humans; however, it does produce an average blood alcohol content of around 100-140 MGN/dl at peak times of consumption (El Shawa et al., 2013). Although this is high in human standards, murine models demonstrate a greater ability to break down alcohol based on a much higher metabolic rate, especially in mice (Cederbaum 2012).

Food and fluid consumption

Food and fluid consumption were measured again each day to assess potential confounding differences between F1, F2, F3 and control dams in terms of caloric intake. The weight of the mouse chow was measured using a standard Fisher Scientific Scale. Liquid intake of water and ethanol solution was measured using a graduated drinking bottle. Throughout the gestation process, each dam's body weight was measured using a standard Fisher Scientific scale daily. The final weight was determined the day before birth. This was used to determine total maternal weight gain.

Breeding of first filial generation mice

The first filial (F1) generation of mice exposed to ethanol prenatally was bred using a maternal model of self-administration of 25% ethanol (El Shawa et al., 2013). Two alcohol naive ninety-day-old female mice were paired with one control male of the same cage in the evening, just before their dark cycle.

Conception is signified by the observation of a vaginal plug. On the day of conception, Gestational day (G) 0.5, pregnant females are moved to a separate cage. Dams were weight-matched, separated into ethanol-treated and control groups, and provided *ad libitum* access to standard mouse chow.

Breeding of second and third filial generation mice

F1 PrEE male offspring generated from female mice that consumed 25% ethanol throughout gestation were used to breed the F2 generation. All mice were weighed on the day of birth and were inspected for any obvious developmental anomalies. PrEE F1 pups were cross-fostered with a control dam. Pairing F1 males with control females produced the F2 generation. Females were separated from the male on the day a vaginal plug was observed. Dams generating the F2 generation were fed standard mouse chow and water *ad libitum*. Breeding of the F3 generation was done in the same way as breeding of the F2 generation. A summary of the breeding paradigm can be seen in Figure 1. Food and fluid intake were monitored to assess any confounding changes in caloric and fluid intake as compared to control type pregnant dams. Weight gain of the dams was also recorded daily until the day prior to birth (Figure 2).

Tissue preparation

Control, F1, F2 and F3 mice were assigned randomly to separate groups and assessed behaviorally with only one test completed on each mouse. After

behavioral testing subjects to be used for P20 Nissl stain analyses or dye tracing

analysis were euthanized with a lethal dose of sodium pentobarbital (100

MGN/kg) and transcardially perfused with 4% paraformaldehyde (PFA) in 0.1 M

phosphate buffer, pH 7.4. The brains were dissected from the skull, weighed,

hemisected, and postfixed in 4% PFA for 6 hours.

INC analysis

To observe INC development in twenty day old F1 mice (directly exposed to

ethanol *in utero* via maternal consumption), F2 mice (descendant from PrEE F1

males) and F3 mice (descendant from PrEE F2 males), crystals of 1,1'-

Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI; Invitrogen) and 4-(4-

(dihexadecylamino)styryl)-N-methylpyridinium iodide (DiA; Invitrogen) were

placed in the developing neocortex. Single dye crystals were placed into two

sensory areas: putative somatosensory cortex or putative visual cortex. The

crystals were inserted into the cortex perpendicular to the surface to a depth

where the tip of the crystal was level with the cortical surface. After dye

placement, the brains were stored in 4% PFA at room temperature for a period of

8-10 weeks to allow for transport of tracer. Labeled cells observed in the dorsal

thalamic nuclei were used as indices for adequate transport of the tracer. Brain

hemispheres were embedded in low-melting point agarose and sectioned in the

coronal plane into 100 um-thick sections using a vibratome. Sections were

counterstained with 4', 6-diamidine-2- phenylindole dihydrochloride crystallized

(DAPI; Roche), mounted onto glass slides, coverslipped with Vectashield

mounting medium for fluorescence (Vector Laboratories), and imaged. The

location of the dye placement was verified for each case using thalamocortical

labeling observed in sections of experimental and control brain hemisections. As

had been done previously (Huffman et al., 2004) putative somatosensory cortex

DPLs were included in our analysis if retrogradely labeled cells were observed in

the ventral posterior (VP) nucleus of the dorsal thalamus. Putative visual cortex

DPLs were included in our analysis if retrogradely labeled cells were observed in

the dorsal lateral geniculate nucleus (dLGN).

Analysis of dye tracings

All experimental and control sections were digitally imaged with three different

filters using a Zeiss Axio Imager Upright Microscope equipped with fluorescence,

and captured using a digital high-resolution Zeiss Axio camera connected to a

PC running Axiovision software (version 4.7). The filters used were as follows:

blue for DAPI counterstain, red for DiI, and green for DiA labeling (excitation

wavelengths: blue, DAPI 359 nm; red, Cy 3 550 nm; green, GFP 470 nm;

emission wavelengths: blue, DAPI 461 nm; red, Cy 3 570 nm; green, GFP 509

nm). The three images were merged and saved in high resolution TIF format.

The amount of dye spread, also referred to as projection zone, was determined

by measuring the extent of retrogradely labeled cells from the DPL as a

percentage of cortical length. The size of the injection site was also determined

as a percent of total cortical length. Fluorescence microscopic images of control

and experimental F1-F3 P0 brains labeled with dye in the putative VSx and SCx

were used to calculate estimated number of labeled cells.

Structural neuroanatomy measurements.

Brain weight and cortical length were recorded post mortem after dissection from

the skull and prior to sectioning for neuroanatomical measurements. Images to

be used for cortical measures were taken from Nissl stained sections. Sections

were measured across all cases using an electronic micrometer in Image J.

Cortical thickness measures included the dorsal frontal cortex, prelimbic cortex,

the somatosensory cortex, auditory cortex, and visual cortex. Thickness was

determined using a line perpendicular to the cortical surface extending from layer

1 to the base on layer 6 just above white matter. Cortical areas were identified

with a mouse brain atlas (Paxinos & Franklin 2004) using anatomical landmarks

such as the genu of the corpus callosum and the fimbria of hippocampus. Sub-

cortical structure measures included the corpus callosum, CA3 sub region of the

hippocampus, dorsal lateral geniculate nucleus, medial geniculate nucleus,

ventral-posterior nucleus, basal ganglia and the amygdala. Corpus callosum

thickness was measured from the midline region. Hippocampal measures were

taken from the CA3 subfield by measuring the thickness of the pyramidal cell

layer. Boundaries for dorsal lateral geniculate nucleus were based on the

intergeniculate leaf for the ventral lateral border, the external medullary lamina

for the ventral medial border, the lateral posterior nucleus for the dorsal border, and the brachium of the superior colliculus for the dorsal lateral border. Boundaries for medial geniculate nucleus (MGN) were based on the superior thalamic radiation as the medial border, the intergeniculate leaf as the lateral border, and the zona incerta as the ventral border. Ventral posterior measures were taken using the medial lemniscus as the ventral border, the external medullary lamina as the dorsal border, and the ventral posterior-lateral nucleus as the lateral border. Measures of basal ganglia included the caudate putamen, globus pallidus, and ventral palladium. Amygdala measures included the central amygdala, basolateral, and basomedial nuclei. Basal Ganglia, amygdala, VP, MGN and dLGN volumes were calculated by drawing borders around the BG and dLGN in serial sections at a fixed magnification using ImageJ, Brains post-fixed in 4% PFA were cryoprotected in 30% sucrose overnight then cut into 40 um thick sections on a cryostat. Sections were then stained following the standard protocol for Nissl staining, cover-slipped with Permount and imaged using a Zeiss Axio camera connected to a Zeiss Discovery.V12 stereomicroscope.

Behavior analyses

To evaluate behavioral variations associated with transgenerational PrEE, we examined anxiety, motor coordination and sensorimotor integration function, depressive behavior, and social behavior in Control, F1, F2, and F3 mice. This

was done through a battery of behavioral assays including: the Suok test, three chambered sociability chamber, elevated plus maze, forced swim test, rotarod test and the adhesive removal test. The behavioral assays require the mice to be of a certain age and size before they can be tested; therefore, behavioral assays were conducted at the earliest available age: P20 in F1, F2, F3, and control mice.

Suok test: The suok test measures an animal's ability to integrate sensory inputs and control motor outputs along with anxiety (Wozniak et al., 2004; Kalueff et al., 2008; Glajch et al., 2012). The Souk apparatus was constructed in accordance with specifications published previously (Kalueff et al., 2008) and consists of a smooth 2-meter long aluminum rod, 3-centimeters in diameter, elevated to a height of 20 centimeters. The tube is divided into 10-centimeter segments by colored markings and held in place between two clear acrylic walls. A 20-centimeter zone is marked at the center most point of the rod and serves as the placement location when starting the assay. All mice to be tested were acclimated to the dimly lit behavioral room one hour before analysis. At the start of each five-minute testing period, animals were placed on the center of the bar with their body parallel to the rod. Mice that fall off the apparatus are quickly placed back onto the rod in the position they fell from. Several measures of behavior are observed and scored by trained observers: horizontal exploration activity, latency to leave the central zone, segments crossed, vertical exploration, which included the number of rears and wall leanings, directed exploration as measured by movements to the side of the bar, grooming behavior, risk

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assessment behaviors, indicated by stretch-attend postures, freezing behavior, and number of defecations. The number of falls and miss-steps is also recorded. Testing sessions are recorded using an HD web camera and saved on a local desktop computer for further analysis. The rod is cleaned with Virkon between cases to eliminate olfactory cues. Reduced scores signify a change in sensorimotor integration and motor coordination, while changes to the latency to leave center and more instances of freezing behavior indicates higher levels of anxiety. All time measures were documented with hand held stopwatches and stereotyped behaviors are recorded during the test.

Sociability test: To evaluate altered social behavior we employed the three-chambered sociability test. On the day of behavioral assessment the animal's cages are placed inside the behavior testing room several hours prior to testing to acclimate. The sociability chamber allows us to examine a subject social behavioral in regards to other mice. The apparatus involves a clear Plexiglas chamber with three rectangular areas divided by clear walls inside the chamber. During the test, two small cages are placed in opposite ends of the chamber; one cup is empty, while the other contains a mouse of similar age that was shown to be docile during pretesting, called the target mouse. By comparing how much time the test animal spends with the empty cup versus the target mouse, we gain insight into its social behavior (Nadler et al., 2004). Due to the social nature of these animals, a control mouse should spend significantly more time near the cage with the target mouse. Prior to the test, the target mice are

assessed and allowed to acclimate to the testing chamber and the cages they will be placed inside. During the test, the experimental animal is first allowed to acclimate to an empty central chamber, then to all three chambers with empty cages placed inside. For the testing period the mouse is placed within the center while the two cages are placed at opposing ends (empty vs. target mouse), the chambers are opened and the animals is observed for ten minutes. The behavior is video recorded and scored later by comparing time spent in each chamber. A standard student's T-test is used to determine the difference in time spent in one chamber versus the other and a ratio of time spent with the mouse versus away from the mouse (in center or opposing chamber) is used to compare differences across groups.

Forced swim test: The forced swim test (FST) is employed to assess depressive-like behavior (Petit-Demouliere et al., 2005). The FST consists of an acrylic cylinder, approximately 30 cm in height and 13 cm in diameter. The cylinder is filled approximately 60% with room temperature water and a video recording camera is placed in front. After habituating to the test room for 1 hour, each test animal is placed within the cylinder and behavior is recorded for 6 minutes. At the end of the testing period, mice were removed from the tank, dried and placed back into their home-cage. Control and experimental animals were tested in the same way with the 6-minute duration. The first two minutes of the test are considered an acclimation phase; scoring of total mobility time is calculated by counting the total time the mouse is mobile and subtracting that

from the test time of 240 seconds. Increased periods of immobility time are inferred to be the result of increased depressive responses. ANOVA was used to compare depressive-like behaviors between control, F1, F2 and F3 animals. An alpha of 0.05 was used to determine significance.

Elevated plus maze: The elevated plus maze provides another method of assessing anxiety like behavior in test animals. The test utilizes a rodent's natural behavior of open area avoidance and to prefer dark enclosed spaces (Pellow et al., 1985; Komada et al., 2008). The elevated plus maze is constructed using plywood and dimensions were a height of 50 cm and consisted of four 54 cm wide arms that are 30 cm long and form a T shape. Two arms were enclosed by 15 cm high-walls while the remaining two arms are open. The maze was placed in the center of the behavioral testing room with a video camera located directly above it. Prior to testing, both control and experimental mice were allowed to habituate to the testing room for one hour. Each test animal was then placed in the center of the elevated plus maze facing an open arm behavior is recorded for a 5-minute period. Using the video recording, time spent in the open arm, closed arm and center, as well as number of arm entries were scored. ANOVAs were conducted to analyze behavioral differences between test animals. *An* alpha *of* less than 0.05 was used to establish significance.

Accelerated rotarod : The accelerated rotarod (AR) behavioral assay allows researchers to assess balance, motor coordination, and learning in mice

(Jones and Roberts 1968; Pritchett and Mulder, 2003). The latency to fall on the first trial between experimental conditions is used to score balance and coordination, while increased latency to fall over time between the first trial and subsequent trials is indicative of motor learning (Lalonde et al., 2003; Buitrago et al., 2004). The AR apparatus (Jones and Roberts) consists of a rotating rod that accelerated from 4 to 40 rotations per minute and is divided into 5 separate lanes. For testing, mice were habituated to the test room for 1 hour, and then scored during 5-minute trial periods. Five total trials were performed with 30-minute rest intervals between each trial. Latency to fall from the bar was recorded for each animal during each 5-minute trial. Repeated-measures ANOVAs with Tukey's post-hoc analysis were used for statistical analysis. An alpha of 0.05 was used to establish significance between groups.

Adhesive removal test: The adhesive removal test (ART) was initially employed using mice to assess sensorimotor deficits following traumatic brain injury (Starkey et al., 2005; Bouet et al., 2007; Freret et al., 2009). Somatosensory and motor functions are evaluated by measuring the time required to remove an adhesive tape strip from the snout (Fleming et al., 2013). Animals were habituated to the test room for one hour. A small adhesive strip (approximately 0.3 cm × 0.3 cm) is placed onto the snout using forceps and the animal is released. Time to remove the strip was recorded. If unable to perform the task within 60 seconds, the strip is removed by the experimenter. All mice received 3 trials, with a 10-minute rest interval between each trial. Repeated-

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measures ANOVA with Tukey's post-hoc analysis were performed. An alpha of 0.05 was used to establish significance.

Statistical Analyses

Results are presented as mean ± S.E.M. ANOVA with Tukey's *post hoc* analysis was used to establish significant differences between dam measures (weight grain, food consumption, liquid consumption, and litter size), pup measures (body weight, brain weight, cortical thickness, neuroanatomical volume, cell packing density), and dye tracing analysis of F1, F2, F3 and control mice. For data displayed as percent change, mean baseline corrected control was set as 100%, with experimental measures expressed as percentage variation from control mean.

Results

Dam measurements

Previous work in our lab found no difference in weight gain or food consumption by the pregnant dams and we concluded no confounding factors such as lowered caloric intake due to PrEE. There were no differences in food intake between control and experimental animals (Figure 2A; control, 6.928 ± 0.314 g/day; F1, 6.987 ± 0.532 g/day, p=0.999; F2, 7.328 ± 0.401 g/day, p=0.914; F3, 7.244 ± 0.434 g/day, p=0.965). Gestational weight gain was significantly reduced in F1–F3 animals when compared to controls (Figure 2B; control, 24.44 ± 1.381 g; F1, 19.70 ± 0.770g, p<0.05; F2, 19.03 ± 0.894g, p<0.05; F3, 18.93 ± 2.153g,

p<0.05); this was accompanied by a reduction in litter size in all 3 experimental generations (Figure 2C; control, 11.250 ± 0.470; F1, 9.043 ± 0.571, p<0.05; F2, 8.750 ± 0.636, p<0.05; F3, 8.250 ± 0.560, p<0.05). Dam plasma osmolality showed no significant differences between groups (Figure 2D; control, 308.2 ± 1.773 mosm/kg; F1, 309.2 ± −1.645 mosm/kg, p=0.977; F2, 310.3 ± 2.161 mosm/kg, p=0.871; F3, 309.5 ± 2.237 mosm/kg, p=0.965). Dam BEC measures revealed blood ethanol only in F1 dams, with an average of 104.4 ± 1.206 mg EtOH/dl at GD9, increasing to 135.2 ± 4.126 mg EtOH/dl at GD19, with no ethanol detected in F2 and F3 dams (Abbott and Rohac et al., 2017).

Pup Measures

Gross anatomical measures of body weight, brain weight and cortical length all revealed persistent changes in the first generation of P20 PrEE mice; however, subsequent generations did not show significant differences (Figure 10 & 11). Body weights of P20 experimental mice were significantly less [$F_{(3,97)}$ = 12.67, *p< 0.0001*] (Figure 10A), as were brain weights [$F_{(3,24)}$ = 5.656, *p = 0.0045*] (Figure 10B). Cortical lengths were also reduced [$F_{(3,20)}$ = 6.808, *p= 0.0024*] (Fig 10C; 11). Post hoc analyses revealed that differences in all three metrics (body weight, brain weight and cortical length) occurred in only F1 generation (Figure 10A-C).

Dye Tracing

INC development was examined across three generations of PrEE mice by placing DiA and DiI crystals in postmortem brains of P20 control, F1, F2 and F3 mice. DiA (green) injections placed into P20 control somatosensory cortex highlights retrogradely labeled cells rostral and caudal (Figure 12 A1-D1) to the dye placement location (DPL, starred, Figure 12 C1). DiI (red) injections placed into P20 control visual cortex also results in retrogradely labeled cells rostral and caudal (Figure 12 C1-F1) to the DPL (starred, Figure 12 E1). No differences were observed between P20 experimental animals of all generations and control mice; similar INC labeling patterns were observed in control, F1, F2 and F3 animals where DiA placement in somatosensory cortex labeled cells rostral and caudal (Figure 12). Thus, the PrEE phenotype that shows ectopic INC development in F1, F2 and F3 generations of mice at P0 is recused by P20 in F1 mice and not present in P20 of the subsequent generations.

Neocortical and subcortical measurements

Measurements taken from P20 experimental cases suggested a strong direct effect of ethanol exposure (F1 generation). Many of these ethanol-induced phenotypes are rescued in the second and third generation.

Frontal cortex: Measures of dorsal frontal cortical thickness showed a significant thickening in experimental animals [$F_{(3,28)}$ = 5.059, p=0.0063] (Figure 13 A; control 100 ± 4.97%, F1 126.9 ± 4.93%, F2 121.5 ± 6.09%, F3 120.5 ± 4.94%).

Post hoc analyses revealed significant differences in all three experimental generations with F1 animals at p<0.01 (Figure 13 A2), and F2 and F3 at p<0.05 (Figure 13 A3-A4).

Prelimbic cortex: Measures of prelimbic cortical thickness were also significantly different for experimental conditions versus controls [F(3,30) = 2.893, p=0.0515] (Figure 13 B; control ± 5.04%, F1 119.9 ± 3.31%, F2 106,4 ±6.41%, F3 109.3 ± 5.17%). Post hoc analyses revealed a significant thickening of F1 prelimbic cortex compared to controls (p<0.05) (Figure 12 B2), but not F2 and F3 animals (Figure 13 B3-B4).

Somatosensory cortex: Measurements of somatosensory cortical thickness showed a significant difference in experimental animals [F(3,30) = 3.836, p=0.0195] (Figure 13 C; control ± 3.52%, F1 121.1 ± 7.18%, F2 99.63 ± 4.09%, F3 104.3 ± 4.65%). Post hoc analyses revealed a significant thickening in F1 somatosensory cortex (Figure 13 C2; p=0.0402), with no difference in F2 and F3 animals compared to controls (Figure 13 C3-C4; F2 p>0.9999, F3 p=0.9462).

Auditory cortex: Measures of primary auditory cortical thickness also revealed a significant main effect in our experimental condition [F(3,28) = 3.935, p=0.018] (Figure 13 D; control 100 ± 3.52%, F1 123.4 ± 7.13%, F2 100.8 ± 5.43%, F3 101.4 ± 3.57%) with post hoc analysis showing F1 animals had a significantly

thicker auditory cortex (Figure 13 D2; p=0.035) compared to controls, with no difference for F2 and F3 animals (Figure 13 D3-D4).

Visual cortex: Finally, measures of visual cortex thickness were not significantly different across conditions [$F_{(3,30)}$ = 1.613, p=0.2071] (Figure 13 E1-E4; control 100 ± 5.48%, F1 93.46 ± 4.47%, F2 108.2 ± 5.66%, F3 102.4 ± 4.13%).

Corpus callosum: Measurements of corpus callosum thickness in experimental and control brains were significantly different [$F_{(3,32)}$ = 4.436, p=0.010] (Figure 14 A; control 100 ± 5.95%, F1 77.17 ± 4.43%, F2 78.23 ± 3.26%, F3 91.45 ± 6.57%), with post hoc analyses showing both F1 and F2 cases were smaller (p<0.05) (Figure 13 A2-A3), while F3 cases showed no difference (Figure 14 A4; p>0.05).

Hippocampus: Measurements of the CA3 region of the hippocampus at P20 revealed a significant thickening within F1 tissue when compared to controls [$F_{(3, 36)}$ = 3.327, p=0.0306] (Figure 14 B; control 100 ± 4.89%, F1 119.9 ± 4.09%, F2 112.5 ± 3.6%, F3 110.0 ± 5.02%). Post hoc analyses revealed no differences between F2, F3 and control cases (Figure 14 B3-B4), while F1 was significant at *p<0.05*(Figure 14 B2).

Dorsal thalamic nuclei: Within the thalamus, dLGN volumetric measures taken

from serial sections revealed significant differences [$F_{(3,28)} = 4.031$, p=0.0168]

(Figure 14 C; control 100 ± 6.12%; F1 77.2 ± 2.97%; F2 83.24 ± 5.53%; F3 83.77

± 4.21%). Post hoc analyses found that F1 animals had reduced dLGN volumes

(Figure 14 C2; p<0.05), but F2 or F3 dLGN volumes were not different compared

to controls (Figure 14 C3-C4). Volumetric measures taken from serial sections of

the MGN suggest no significant difference between control and experimental

animals [$F_{(3,16)} = 0.2563$, p=0.8557] (Fig 14 D1-D4; control 100 ± 6.93%; F1

102.3 ± 4.30%; F2 98.48 ± 10.56%; F3 92.57 ± 9.82%). Volumetric measures

taken from serial sections of the VP nucleus showed no differences between

control and experimental groups at P20 [$F_{(3,20)} = 2.113$, p=0.1306] (Figure 14

E1-E4; control 100 ± 7.67%; F1 109.9 ± 5.59; F2 110.3 ± 4.65%; F3 93.45 ±

3.85%).

Amygdala: Finally, no differences were observed for the amygdala when

measuring volume based on serial sections within all three generations at P20

[$F_{(3,19)} = 0.4234$, p=0.7384] (Figure 15 A1-A4; control 100 ± 9.87%; F1 87.87 ±

5.92; F2 95.14 ± 7.62%; F3 91.43 ± 8.35%).

Basal ganglia: Additionally no difference was detected for measurements of the

volume of the basal ganglia in control, F1, F2 or F3 animals [$F_{(3,22)} = 1.207$,

p=0.3304] (Figure 15 B1-B4; control 100 ± 5.88%; F1 90.56 ± 4.61; F2 86.48 ±

4.68%; F3 91.47 ± 4.77%).

Behavioral Assays

Behavioral analyses revealed PrEE induced alterations in sensory-motor

integration, anxiety, social behavior, depressive behavior and risk-taking

behavior.

Suok bar: Using the suok test we can evaluate correlates for anxiety and motor

development (sensorimotor integration and motor coordination) in a single assay.

Significant differences were observed across generations when compared to

controls. F1 and F2 mice displayed greater anxiety revealed by an increased

average latency to leave the center of the suok bar versus control mice (Figure

16 A, Control = 3.93 ± 0.626s; F1 = 22.45 ±2.917s, *p<0.05;* F2 = 31.06 ± 5.737s,

p<0.0001); however F3 mice showed no difference from controls (F3 = 6.45 ±

1.136s). Both F1 and F3 mice showed fewer stereotyped rearing/grooming

events when compared to controls (Figure 16 B, control = 1.750 ± 0.1927; F1 =

0.9412 ± 0.1044, p<0.05; F3 = 0.9375 ± 0.1930, p<0.05), also an indicator of

increased anxiety. F1, F2 and F3 mice showed a reduction in the mean number

of exploratory behaviors as they traversed the suok bar (Figure 16 C, control =

72.38 ± 3.245; F1 51.88 ± 2.89; F2 = 59.97 ± 1.961; F3 = 59.06 ± 5.33). F1, F2 and F3 mice made significantly more missteps on the suok bar than control animals (Figure 16 D, Control = 9.968 ± 1.120; F1 =22.29 ± 1.808, p<0.0001; F2 = 23.09 ± 1.744, *p<0.0001*; F3 = 18.8 ± 0.8814, p<0.01). F2 and F3 mice also fell off of the suok bar more than control animals (Figure 16 E, Control = 1.042 ± 0.2854; F1 = 4.647 ± 0.8087, p<0.001; F2 = 3.429 ± 0.4697, p<0.01; F3 = 3.25 ± 0.5809, p<0.05). Segments crossed, a measure of the total distance travelled, was not different between F1, F2, F3 and control animals (Control = 87.46 ± 5.015; F1 = 98.53 ± 10.01; F2 = 80.00 ± 7.434; F3 = 108.7 ± 4.773).

Three-chambered sociability test: The sociability test allows for evaluation of social interactions between different groups. For this assay, control mice spent significantly less time in the empty chamber as apposed to the chamber with the target mouse (t =3.012, df=14, p=0.0093). The F1, F2 and the F3 generations did not mimic this behavior. The ratio of time spent with the mouse versus time away from the target mouse can be seen in Figure 16 F, and demonstrates that the experimental generations spent significantly less time with the target mouse than in other parts of the apparatus [F(3,24) = 3.720, p=0.0250]. Post hoc analysis revealing all three generations spent significantly less time as a ratio with the target mouse compared to control animals (p<0.05).

Rotarod: The accelerated rotarod was used to observe and score motor coordination and learning deficits between control, F1, F2 and F3 mice. Experimental animals were significantly less proficient in motor coordination and demonstrated learning deficits across trials when compared to their control counterparts (Figure 17 A) ($F_{(3,32)}$ = 9.146, $p<0.0002$). Post-hoc analyses revealed trial 1 and trial 2 to be the main source of variation between experimental and control animals. Measures of time spent on the rotarod demonstrate that during trial 1 control animals maintained their balance on the rotarod for significantly more time compared to all three experimental generations (control: 228.3 ± 18.29 sec; F1 108.9 ±7.228 sec; F2 151.6 ± 21.76 sec; F3 155.6 ± 23.67 sec). Measures from trial 2 demonstrate a similar trend (control: 283.2 ± 27.47 sec; F1 106.6 ± 5.93 sec; F2 145.4 ± 22.82 sec; F3 172.6 ± 21.68 sec). Measures from trials 3 and 4 suggest the differences between control and experimental animals were no longer significant.

Adhesive removal test: Sensorimotor integration was also measured using the adhesive removal test by scoring the time required for animals to remove an adhesive strip from their snout. Latency to remove the strip was significantly different between groups, with experimental generations showing latencies in strip removal (Figure 17 B) [$F_{(3,40)}$ = 5.029, $p<0.0047$]. Post-hoc analyses revealed trial 1 and trial 2 to be the main source of variation between experimental and control animals ($p<0.05$).

Elevated plus maze: Additionally, we assessed behavior using the elevated plus maze. When comparing the amount of time spent in the open arms of the maze, F1, F2 and F3 statistical analysis suggested a significant difference between conditions [$F_{(3,28)} = 4.517$, $p=0.0105$] (Figure 17 C). *Post hoc* analyses revealed that all three generations were significantly different and spent more time in the open arms of the maze ($p<0.05$).

Forced swim test: Depressive behavior was measured using the forced swim test. Significantly greater depressive behavior was observed in the F1, F2 and F3 generations when compared to controls (Figure 17 D). ANOVA conducted on the time spent immobile within the swim chamber revealed significant group differences [$F_{(3,28)} = 4.647$, $p=0.0093$]. Post hoc analyses revealed all three experimental generations to be different compared to controls ($p<0.05$, F1-F3) and spent more time immobile in the swim chamber.

Discussion

This study sought to discover whether the transgenerational changes observed in newborn PrEE mice would persist through the early postnatal period. Many of the changes we found at birth were still present in the first generation at twenty days old, but were not in the second and third generations. Most importantly, however, was that the behavioral assays done at all three generations demonstrated stark

differences in those animals descended from prenatally exposed male mice. Suggesting, that we need further study to find exactly what is mediating the behavioral differences.

As the brain develops, billions of neurons make many synapses and become interconnected in eventually precise circuits. The initial pattern of connections, however, is most often imprecise. The refinement of these neural networks is an activity dependent process that, when allowed to progress normally, results in very precise networks of connections observed in adults (Goodman et al., 1993; Katz et al., 1996). The activity dependent mechanisms that form the specific patterns of connectivity are important and may mediate some of the damage observed in PrEE newborn brains. PrEE has the potential to alter neuronal migration and ultimately affect postnatal brain growth (Miller et al., 1993). Together, this suggests that the brain is far from complete at birth and the reorganization and refinement of cortical areas may be susceptible to disruption by prenatal ethanol exposure. It also suggests that the activity dependence mechanisms may be able to provide some corrective action toward refinement of connections, since they are never perfectly accurate in the first place.

Gross anatomy of PrEE P20 mice

Many of the changes observed in the newborn mice were observed in the first generation (F1) P20 PrEE mice, but did not persist into the second and third generations. Body weight, brain weight and cortical length all were significantly

reduced in F1 P20 mice, but F2 and F3 animals were not different from control cases on average. This suggests a strong direct effect of exposure, but some mediating effect that rescues the phenotype, at this gross level of analysis, in subsequent generations. This is important and suggests that although we can measure several changes out across generations at birth, activity dependent mechanisms may be ameliorating the alterations through development.

Neuroanatomy in P20 mice

Several of the anatomical changes documented in newborn PrEE mice continued to be observed in twenty-day-old mice. Specifically, dorsal frontal cortex continued to be thicker in experimental animals compared to controls at all three generations. Multiple studies have demonstrated the sensitivity of frontal cortex to ethanol insult (Sowell et al., 2008; Yang et al., 2011). Results from this study suggest a persistent change that may be mediated by epigenetic factors, but doesn't appear to be due to cell proliferation, as cell-packing density in cortex was not different between control and experimental groups (Table 3). The corpus callosum also showed multi-generational changes due to PrEE, but only into the second generation. The effects seen here may be due direct fetal exposure of F1 tissue and exposure of germ cells within the F1 embryo that are passed onto the F2 generation.

Exposure to ethanol during pregnancy changes the development of sensory areas in the brain, potentially altering the processing of sensory input.

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Visual and somatosensory areas appear particularly sensitive to ethanol insult. A study by Lantz and colleagues in 2014 suggested that PrEE reduces the refinement and accuracy of connections and electrophysiological responses in primary visual cortex. Altered cell morphology and thalamic alterations have also been shown to result from PrEE (Mooney et al., 2010; Church et al., 2012; De Giorgio and Granato, 2015). Together, these results suggest strong alterations in the networks responsible for processing and integrating primary sensory information including visual, auditory and somatosensory input. Many of the areas targeted for anatomical measurement in this study showed changes in the first generation PrEE mice, but were no longer different in second and third generations. The somatosensory cortex, auditory cortex, prelimbic cortex, dorsal lateral geniculate nucleus, and area CA3 of the hippocampus were all different in the first generation of P20 mice, but not in subsequent generations. This may suggest differences in sensitivity to ethanol insult or may be the product of corrective activity occurring as the brain develops. Further study would be needed to determine why some areas appear more effected than others or we may need more refined methods of detecting persistent changes that do exist at P20, but are not detected by the methods used in this study.

The disrupted intraneocortical network discovered in PrEE F1-F3 newborn mice was not observed in the three generations of PrEE P20 mice (Abbott et al., 2017). This is interesting and may be the result of natural pruning. The normal development of the nervous system results in an abundance of connections,

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many of which are unnecessary and are later pruned (Cowan et al., 1984). The aberrant connections observed in newborn PrEE mice across generations would be ideal candidates for pruning, as they are never documented in normal developing animals and are unlikely to be useful connections.

Behavior

One of the main reasons we investigated the neuroanatomy at P20 was due to the behavioral differences observed in our initial alcohol study (El Shawa et al. 2013). Many of our behavioral tests are performed at P20 and most show strong behavioral alterations compared to control animals. In this study we found altered social behavior, motor control and sensorimotor integration using multiple tests, altered anxiety and increased depressive behavior. The fact that many of the areas we measured were not different in second and third generation animals, despite the behavior being altered implicates a couple possibilities. First we may not be targeting the correct areas to highlight the source of the aberrant behavior; second, there may still be persistent changes, but at much smaller resolutions than is detectable by the methods employed here. What is clear is that PrEE in one generation is having long lasting changes in the behavioral development in subsequent offspring. In an attempt to combat the first possibility we targeted primary sensory areas across the neocortex including somatosensory and visual areas in an attempt to canvas the brain and capture those areas that may mediate such behavior. More study is necessary to discover the mechanisms underlying the behavior observed in these animals.

Conclusions

The results of this chapter suggest a persistent phenotype that is measureable

through development and across at least three generations. The dorsal frontal

cortex was significantly thicker, which corroborates human FASD measures

(Fernández-Jaén et al., 2011) and may contribute to the behavioral deficits

observed in P20 animals (Berger et al., 1997). In our model of FASD, using CD-1

mice, sensorimotor dysfunction, altered anxiety, altered social interaction and

depressive-like behaviors are not only detectable in PrEE mice directly exposed

to ethanol (F1 and F2), but are also measureable in unexposed (F3) offspring.

Data from this study along with other recently published work (Abbott et al.,

2017) suggest that prenatal ethanol exposure dramatically alters nervous system

development resulting in a wide array of dysfunctional behaviors. The fact that

some of the areas measured (anatomy and INCs) were no longer different in

second and third generations suggests some level of correction is possible after

prenatal ethanol exposure, at least at this level of analysis; however, the end

result of altered behavior did not show any measureable improvement across

generations.

Chapter 3: Perceived safety of common food, beverage and medication items during pregnancy

Introduction

Fetal outcomes are crucially linked to the mother's consumption habits during pregnancy (Kaiser et al., 2010; Keen et al., 2003). For instance, the scientific study of prenatal ethanol, or alcohol, exposure (PrEE) has shown convincing evidence for negative outcomes for the child (Spohr et al., 1993) and published work by our laboratory has demonstrated deleterious phenotypes due to PrEE in animal models (El Shawa et al., 2013; Abbott et al., 2016). However, the general public's assessment of what may or may not present a risk to the fetus does not always reflect the most accurate or up-to-date scientific information. Thus, it is important that we understand commonly held perceptions regarding the safety of certain foods, beverages, and medications during pregnancy. Preconceived ideas based on non-medical sources have the potential to be strong guiding mechanisms that produce unhealthy and unsafe behavior during pregnancy, yet there remains a paucity of research on the influence of such perceptions on actual consumption habits. Not only will the assessment of the general public's perception of safe consumption habits during pregnancy highlight gaps between scientific understanding and public knowledge, it will also inform future research on the extent to which misinformation and misguided beliefs compromise patient compliance with dietary directives set by medical professionals. As

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misinformation about the apparent benefits of certain food items can spread and persist, be it alcohol or otherwise, we must ensure that the general pubic is aware of the conclusions reached by scientific research.

Fetal development is an exceptionally sensitive period for toxic insult. Exposure to toxins can induce developmental changes in the body and especially the brain that can lead to permanent cognitive and behavioral pathologies. Exposure to certain substances, both legal and illegal, has been shown to cause both short and long term effects. Illegal substances like cocaine, heroine, and methamphetamines have severe teratogenic effects that have been known for some time (Lidow et al., 1995; Chang et al., 2004; Lu et al., 2012), while the deleterious effects of legally prescribed and over-the-counter medications have only recently been probed. According to the CDC: prescription pain killer use has been on the rise. A recent study found that between 28% and 39% of women at reproductive age (15-44) filled a prescription for an opioid medication, with differences based on insurance (CDC 2015). A population-based study conducted by the CDC found several birth defects were correlated with opioid use during pregnancy including: spina bifida, hydrocephaly, glaucoma, gastroschisis, and congenital heart defects (CDC 2015). Additional studies have concluded that taking opioids during pregnancy might also cause preterm birth (before 37 weeks' gestation) and neonatal abstinence syndrome (NAS) (ACOG 2016).

Lesser-known chemicals in foods such as methyl mercury (MeHg) and polychlorinated biphenyls (PCB) (Grandjean, et al. 1995; Ribas-Fito, et al., 2001) have been shown to cause detrimental outcomes for the child. Exposure to MeHg and PCBs is mainly through the consumption of seafood (WHO 1990, Fein et al., 1984). MeHg can cause psychomotor and cognitive dysfunction, while exposure to PCBs results in lower than expected birth weight and head circumference (Vahter et al., 2000). Finally Bisphenol-A (BPA) has been a known teratogen that was removed from infant's plastic bottles due to its harmful effects (Braun, et al., 1998; Vom Saal et al., 2009), yet this chemical still exists in the lining of many canned food items. Exposure to BPA can result in behavioral and reproductive abnormalities (Kundakovic et al., 2013).

A study published in our lab demonstrated that, despite the abundance of nutritional and development studies underscoring the importance of healthy dietary choices during pregnancy, a proportion of pregnant women nonetheless consume substances that are potentially harmful to their baby (Santiago et al., 2013). A person's belief of what food is safe can have a profound impact on their dietary choices during pregnancy. The theory of planned behavior (TPB) postulates that behavior may be best predicted by several factors including personal attitudes (beliefs) toward the behavior, subjective norms, difficulty of the task and volitional control (Ajzen, 1991). The TPB has much correlational evidence supporting it (Albarracin et al., 2001) and intervention studies support the theory by showing that changing patient's TPB constructs changes behavior

(Jemmott et al., 2011). Given the complexities inherent in this kind of decision making, understanding common perceptions of the risk presented by certain foods, beverages, and medications is paramount, as such perceived risks may directly influence choices of consumption during pregnancy.

Materials and Methods

Questionnaire

The Belief about the Nutrition of Pregnant Women (BNPWS) survey was designed to explore commonly held beliefs about the consumption (eating and drinking) habits of pregnant women. The BNPWS takes approximately forty minutes to complete and contains demographic information (ethnicity, age gender, year in school). To explore beliefs a Likert scale style series of questions was used to explore people's perception of how safe each item is for pregnant women to consume. For example: "A woman eats chicken during pregnancy. Please provide your opinion about how safe this is for her developing baby". Food items included: red meat, chicken, tuna, salmon, canned food, sweet desserts, fast food and fresh fruit. Beverage surveyed included: tap water, energy drinks, milk, juice, regular coffee, tea (black or green), colas, wine, beer and mixed alcoholic drinks. Medications surveyed included: non-prescription cold medication, aspirin, Tylenol, Ibuprofen, prenatal vitamins, anti-depressants, and prescription pain medication. Additional questions were asked about the safety of each of the items during each trimester. The BNPWS is not a complete dietary survey, it is designed to be a relatively short questionnaire regarding commonly

consumed products and medications. Meal size was not surveyed. Based on published work in our laboratory on an animal model of FASD our initial interest was to explore alcohol consumption during pregnancy; however, we extended our list to include potential and known teratogens from commonly consumed food and beverage items as well as medications to complete our survey. Non-threatening wording was used and the method of preparation for each item was not assessed.

Participants and procedure

Participants consisted of 697 undergraduate students who were recruited using the psychology department subject pool at a public university in Southern California. Participants completed the survey online using Survey Monkey software. Frequency statistics for the data were presented in tables and differences between were analyzed using non-parametric Kruskal-Wallis tests. Ordinal regression analysis was conducted with gender and ethnicity used as predictor variables to assess whether these two factors alone, or combined, could predict a participant's responses to each question. Statistical significance was set at $p < 0.05$. All statistical analysis were obtained using SPSS version 24.0. This study was conducted in strict adherence with the protocol guidelines approved by the Human Research Review Board at the University of Riverside, CA.

Results

Demographics and participant information

Among the 697 participants, 645 completed the entire questionnaire correctly while 52 were disqualified due to incompleteness, generally due to incomplete surveys. The subject population was 68% female (440), while 32% (204) were male. All participants that completed the survey were over 18 years of age, with 634 (98.3%) between the ages of 18 to 25, 5 between the ages of 26 to 29 (0.8%), 1 participant between 30 and 35 (0.2%) and 1 participant over the age of 40 (0.2%). The subject population was made up of 32 African Americans (5%), 278 Asian/Pacific Islanders (43.1%), 222 Hispanic (34.4%), 30 Middle Easterners (4.7%), and 83 identified as White (12.9%). Of the participants, 632 (98%) did not already have children, 9 participants had at least one child (2%) and 1 participant was pregnant when they took the survey. For a summary of demographics see Table 4.

Perception of safe items

Participants were asked if they thought a selection of food, beverage and medications were safe to consume for a developing baby during pregnancy. Responses were measured using a Likert scale ranging from unsafe, somewhat unsafe, somewhat safe and safe or no opinion. The total number of respondents for the perception of food, beverage and medications is broken down based on ethnicity and gender within Tables 5 - 7.

3.2.1 Food items

A summary of the general perception of food items can be seen in Figure 18, with the items listed from least to most safe based on perception scores. Of the participants that completed the survey 52.9% thought red meat was at least somewhat safe (combined safe and somewhat safe), while 31.9% listed it as at least somewhat unsafe (combined unsafe and somewhat unsafe) (Table 8). Most participants thought red meat was safe to eat up to 1 to 3 times per week during each of the three trimesters with roughly 30% or greater responding red meat was safe to consume during all three trimesters.

A large proportion of participants thought chicken was safe to consume during pregnancy with 62.5% and 25.7% responding it was safe and somewhat safe during pregnancy respectively. A small number thought chicken was somewhat unsafe (2%) while less than 1% thought it was unsafe. Approximately 30% of participants thought chicken was safe to consume between 4 and 6 times per week while a larger percentage thought it was safe 1 to 3 times per week for each trimester: 37.7%, 41.9% and 37.4% for the first, second and third trimester respectively (Table 8).

Participants were evenly distributed on the perception of tuna consumption. Roughly 20% thought it was safe, somewhat safe, somewhat unsafe and unsafe (18.9%, 21.6%, 22.5%, and 22.8% respectively). A third of participants thought tuna could be consumed up to 1 to 3 times per month with

between 20 and 25% feeling it could be consumed 1 to 3 times per week during each trimester. Greater consumption was perceived as unsafe (Table 8). Salmon appeared to have a similar perception as tuna, with approximately 20% of people thinking it was safe, somewhat safe, somewhat unsafe and unsafe (23.3%, 26.2%, 18.4% and 20% respectively). As with tuna, just over 30% of participants thought it could be safe to consume 1-3 times per month and 25% to 30% perceiving it was safe 1 to 3 times per week for each trimester (Table 8). Approximately 15% of participants thought canned food was safe overall, while 29 and 30% thought it was somewhat safe and somewhat unsafe respectively. Canned foods were perceived as unsafe by 12%. Between 65 and 70% of participants thought canned foods were safe to consume up to 1 to 3 times per week during all three trimesters. Fewer participants thought canned foods were safe as the pregnancy progressed with 6%, 4% and 5% perceiving it as unsafe during the first, second and third trimester respectively (Table 8).

Approximately 34% and 35% thought sweet desserts were safe and somewhat safe to consume during pregnancy, while less than 2% perceived them as unsafe. Between 70% and 75% thought they were safe to consume up to 1 to 3 times per week for each of the three trimesters. Less than 8% thought they were safe for each trimester more than 7 times per week (Table 8). Fast food had a much lower perception of safety during pregnancy. Approximately 25% categorized it as unsafe and 43.3% said it as somewhat unsafe. Close to 20% said it was somewhat safe and only 5% placed it as safe.

More than half of participants categorized fast food as only safe to consume 1 to 3 times per month during each trimester (Table 8).

Most participants categorized fresh fruit as safe (93%) with more than 70% placing it as safe to consume more than 7 times per week (Table 8).

Beverages

A summary of the general perception of beverages can be seen in Figure 19, with the beverages listed from least to most safe based on perception scores. 43.7% listed tap water as at least somewhat safe (combined somewhat safe, 23.4% and safe, 20.3%) to consume while 44.8% listed it as at least somewhat unsafe (combined somewhat unsafe, 26.5% and unsafe, 18.3%). Close to a quarter of participants placed tap water as not safe during any of the three trimesters while a similar percentage said it was safe to drink more than 7 times a week.

Most participants thought milk (68.7%) and juice (62.8%) were safe to consume during pregnancy. 22% and 27% thought milk and juice were somewhat safe respectively, while 4.4% listed milk less than somewhat safe, and less than 3% listed juice as less than somewhat safe. Most thought they both was safe several times per week with 30% categorizing each as safe up to 7 times per week.

Colas were listed as safe to consume during pregnancy by approximately 3%, while 30% and 42% listed it as unsafe and somewhat unsafe respectively. Between 43% and 47% listed colas as safe to consume 1 to 3 times per month

for all three trimesters, while between 32% and 37% categorized colas as never safe during the three trimesters (Table 9).

Almost 78% of those surveyed thought energy drinks were not safe to consume during pregnancy. This response did not change when asked about specific trimesters with 78% saying it was not safe during the first and more than 80% categorizing it as unsafe during the second and third trimester. 12.6% listed energy drinks somewhat unsafe, 2.9% said it was somewhat safe and finally 1.2% perceived them as safe (Table 9).

Almost half of those surveyed classified regular coffee as unsafe (47.6%) while 35% said it was somewhat unsafe. More than 30% then said it was safe to drink 1 to 3 times per month during the first two trimesters with a smaller group (28%) listing it was safe 1 to 3 times per month in the third trimester. More than half of participants continued to categorize regular coffee as never safe to consume during each trimester (Table 9).

Almost 30% said black or green tea was safe to consume (29.8%) and 31.6% said it was somewhat safe. A smaller percentage thought it was somewhat unsafe (15%) and 7% listed it as unsafe. The largest percentage of participants thought tea was safe to consume 1 to 3 times per week (28-30%) throughout the pregnancy with 12% to 14% listing it as never safe (Table 9). Most participants (93%) classified beer as unsafe during pregnancy, 0.3% as safe and 0.6% as somewhat safe. More than 90% categorized beer as never safe during all three trimesters, while 4% said it was safe 1 to 3 times per month

during the fist trimester and 3% continued to say 1 to 3 times per month was safe for the second and third trimesters (Table 9).

Wine was listed as safe by 1.1% as somewhat safe by 5.4%, somewhat unsafe by 14.6% and unsafe by 73.3%. Although most participants thought wine was unsafe to consume during pregnancy, more than 14% still listed wine as safe to consume 1-3 times per month during the last two trimesters and 17% listing 1-3 times as safe during the first trimester (Table 9).

A large majority of participants listed mixed alcoholic drinks as unsafe during pregnancy (96%), while 0.3% listed it as safe and 0.5% as somewhat safe. More than 95% listed alcoholic mixed drinks as never safe to consume during all three trimesters (Table 9).

Common medications

A summary of the general perception of medications can be seen in Figure 20, with the medications listed from least to most safe based on perception scores. Less than 2% classified non-prescription cold medications (NPCM) as safe during pregnancy while 36% and 43% listed NPCMs as somewhat unsafe and unsafe respectively. More than 50% then listed NPCMs as never safe during each trimester with the next largest group (37%, 34% and 32% respectively) listed them as safe 1 to 3 times per month during the first, second and third trimester (Table 10).

Tylenol was listed as unsafe and somewhat unsafe by 26% and 36% of participants respectively while approximately 47% to 48% listed it as safe only 1

to 3 times per month during each trimester. 26, 38 and 42, A large percentage listed it as never safe during the first (26%), second (38%) and third (42%) trimester (Table 10).

Ibuprofen was listed as unsafe by 40% of participants, with 33% listing it as somewhat unsafe and 20% and 4% listing aspirin as somewhat safe and safe respectively. When asked per trimester between 43 and 45% listed Ibuprofen as safe 1 to 3 times per month; the next largest group listed it as never safe for all three trimesters.

Participants listed aspirin as unsafe by 35% and 30% listed it as somewhat unsafe. Between 42% and 43% thought aspirin was safe 1 to 3 times per month, while 44% listed it as never safe during the first trimester and 47% listing it as never safe during the second and third trimester (Table 10). A majority of those surveyed thought prenatal vitamins were safe during pregnancy (64%), while only 3% thought they were somewhat unsafe and unsafe (Table 10).

Most participants thought anti-depressants were unsafe to take during pregnancy (65%), less than 1% listed it as safe. More than 70% listed it as unsafe during each of the three trimesters while 19% thought it was safe 1 to 3 times during the first and 16% during the second and third trimesters (Table 10). The largest percentage of participants categorized prescription pain medication (PrPM) as unsafe (35%); however 11% and 20% listed PrPMs as safe and somewhat safe respectively while 24% listed it as somewhat unsafe. A larger

percentage of participants (40%, 42% and 45% for 1st, 2nd and 3rd trimester respectively) listed PrPMs as never safe when asked about each trimester specifically; however, approximately between 15% and 17% still listed them as safe to take 1 to 3 times per week during each trimester. Less than 2% thought they were safe more than 7 times per week (Table 10).

Gender and ethnicity differences

Non-parametric Kruskal-Wallis tests were performed to explore differences in perception between men and women. Kruskal-Wallis tests revealed significant differences in responses between men and women in the following items: tuna (x^2 = 5.329, p=0.021 with a mean rank for men= 346.83 and women= 311.22), tap water (x^2 = 5.038, p=0.025 with a mean rank for men= 346.09 and women= 311.56), energy drinks (x^2 = 6.104, p=0.013 with a mean rank for men= 341.66 and women= 313.62), wine (x^2 = 11.886, p=0.001 with a mean rank for men= 351.82 and women= 309.14), coffee (x^2 = 6.007, p=0.014 with a mean rank for men= 346.80 and women= 311.23), cold medication (x^2 = 6.789, p=0.009 with a mean rank for men= 348.65 and women= 310.38), Ibuprofen (x^2 = 5.065, p=0.024 with a mean rank for men= 345.84 and women= 311.68), and aspirin (x^2 = 5.328, p=0.024 with a mean rank for men= 346.35 and women= 311.44). In general men reported these items as safer than women did on this survey (Figure 21).

Using a linear regression model, with an ordinal scale, we found no significant effect of ethnicity, or gender x ethnicity as a predictor of the

participant's responses to the questions regarding the perception of food, beverage or medication safety.

Discussion

In this study we sought to understand what respondents perceive as safe nutritional habits during pregnancy including food, beverage and medication. This is crucial, as making poor choices during pregnancy can result in life long complications for both the mother and the baby. This study begins to define the differences in public opinion of safe pregnancy related dietary behavior compared to the most recent research conclusions. Once the gaps in public knowledge and perception are identified, efforts can be made to increase education about safe lifestyle choices during pregnancy.

Healthy items

Foods: Items included in our survey that are considered healthy dietary choices were beef, chicken, and fresh fruit. Although there are some health risks associated with hormone and antibiotic levels in meats (Moats et al., 1996), the choline supplementation these items provide has been considered to outweigh the risks, as choline deficiency can cause early developmental issues (Albright et al., 1996). The majority of participants appear to recognize the safety of milk and juice, with more than 60% listing them as safe and more than 80% listing them as at least somewhat safe. The majority of respondents also considered chicken

and fresh fruit safe, and there is little, if any, research to suggest a high risk to maternal or fetal health due to chicken or fruit consumption.

Beverages: The high sugar content of some juices may pose a problem concerning blood glucose management and gestational diabetes mellitus (Casey et al., 1998); however a specific type of juice was not specified and may have affected responses on the survey. Additionally, the vitamins and other nutrients from juices may be a better alternative to other sweetened beverages. Milk is generally accepted as safe by the scientific literature and low intake is associated with possible intrauterine growth reduction (Ludvigsson et al., 2004). According to this survey, the general understanding of the safety of these products agrees with the scientific literature.

Medications: The use of prenatal vitamins, which is recommended by the CDC (Scholl et al., 1997), was considered as healthy supplements for the purpose of the survey and respondents generally agreed with this .

Unhealthy items

Items associated with higher risk during development included: tuna, salmon, canned foods, sweet desserts, fast foods, tea, coffee, energy drinks, beer, mixed drinks, wine, cold medication, Tylenol, Ibuprofen, Ibuprofen, Prescription pain medication, and anti-depressants.

Foods: Studies have suggested tuna and salmon, especially farm-raised salmon, can contain high levels of both methyl mercury and PCBs (Clarkson 1990).

Methyl mercury is thought to accumulate in the fetal brain more readily than the mothers and can have detrimental effects on neurodevelopment altering cell fate, neuron outgrowth, proliferation and migration (Yang, et al., 1973; Choi et al., 1989; Guzzi et al., 2008). Small levels of consumption in children can increase blood mercury levels above the health limit (Hightower et al., 2003). Although mercury levels can vary, the FDA has shown that canned tuna can contain up to 1.816 ppm. The current recommendations by the FDA and the EPA are that pregnant women consume less than 6 ounces of tuna per week (approx. 1 can) and new consumer reports suggest that eating 2.5 ounces per week can cause blood mercury levels to exceed EPA safety standards (Consumer report 2011).

Salmon was perceived as somewhat safer according to the survey, with almost half of respondents listing it as at least somewhat safe. PCBs found in salmon are associated with lowered birth weights, smaller head circumference in newborns and mental impairments in older children (Fein et al., 1984; Patandin et al., 1999; Jacobson et al., 2003). The FDA limits PCBs in fish to 2 ppm; however, studies suggest exposure to even low levels can be clinically relevant (Govarts et al., 2012). These results indicate a need for greater education about the risks of fish consumption during pregnancy.

Canned foods commonly contain BPA in the plastic coating that lines the sides of the metal cans. BPA is an endocrine disrupter that can pose a danger to a developing fetus if consumed in high amounts (Vandendberg et al., 2007). BPA exposure prenatally was associated with an increased risk of ADHD-hyperactivity

symptoms and can cause hormone regulation differences in developing children (Casas et al., 2015; Braun et al., 2011; Howdeshell et al., 1999). The FDA has banned the use of BPA in infant bottles and cups (Vandendberg et al., 2007; Kubwabo et al., 2009), but it remains in the lining of canned foods. Several studies have demonstrated BPA leaching into the foods within the cans (Brotons, et al., 1995; Yoshida et al., 2001; Vandenberg et al., 2007). The results of this study pinpoint a worrying percentage of the population that thought canned foods were safe during pregnancy. Thus, this represents a possible opportunity for educational interventions that may translate to decreases in maternal BPA ingestion.

Frequent consumption of sweet desserts can lead to the development of gestational diabetes mellitus (GDM), which has been associated with several developmental outcomes including larger infants, macrosomia and increased need for cesarean delivery (Casey et al., 1998; Major et al., 1998; Rosenberg et al., 2005; Zhang et al. 2006; Lenders et al., 1994).

A larger percentage of participants thought fast food was relatively unsafe with more than half listing it as safe only 3 times per month. This is interesting and appears to agree with the actual consumption reported in our previous study of pregnant women's eating habits, with 48% reporting consuming fast food 1-3 times per month (Santiago et al., 2013). Frequent consumption of high fat foods during pregnancy can lead to excessive gestational weight gain, which has been associated with several complications during pregnancy including an increased

chance of cesarean birth and preeclampsia (Juhasz et al., 2005; Rosenberg et al., 2005). Infants born to women who gain weight excessively during pregnancy are more likely to be born preterm (Schieve et al., 1999) and be macrosomic at birth (> 9 lbs) (Sewell et al., 2006). It is interesting to note that sweet deserts were perceived as safer that fast food which is high in fat and salt. This may provide support for the effectiveness of the sugar industries attempts to block or hide research that paints their product in a negative light (Kearns et al., 2016).

Beverages: The original purpose of the study was to determine the perceptions of the safety of alcohol during pregnancy. The majority of participants did categorize alcohol, whether it is wine, beer or mixed drinks, as unsafe. Wine had the largest percentage of participants stating it was at least somewhat safe. Additionally, more than 17% listed wine as safe to consume 1 to 3 times per month in the first trimester and 14% for the second and third. Beer and mixed drinks scored much lower on safety. Additionally, wine comparatively scored safer to consume during pregnancy than energy drinks within the survey. The data suggests that although a majority of those surveyed understand the dangers associated with prenatal alcohol exposure, a sizeable group does not. Even a small percentage of people that ignore the risks can result in a large number of FASD cases. The fact that 17% surveyed listed wine as safe 1-3 times per month during the first trimester still shows a strong misunderstanding of the conclusions by scientists and health organizations that no amount of alcohol is safe during pregnancy. Finally, responses indicated that participants scored alcohol as safest

to consume during the first trimester. This is concerning as exposure during the first two months correlates highly with fetal alcohol symptoms (Day et al., 1989). We also included additional questions regarding alcohol and its use during pregnancy aimed at understanding the interplay between perceived risk, outside influences, and hypothetical behaviors concerning alcohol usage during pregnancy. One question asked participants to report their hypothetical behavior regarding alcohol consumption if they were to find out they or their partner were pregnant. For this question 95% of respondents said they would cut out all alcohol from their diet, but almost 5% said it would still be acceptable to have the occasional drink (with wine listed within the pre-written response). Additionally the largest group of respondents said they thought 10% of women continued to use alcohol during pregnancy. This is slightly higher than the approximately 6% of women who said they consumed alcohol during pregnancy in our previous study (Santiago et al., 2013). Interestingly, when respondents were asked if they thought medical professionals exaggerated the dangers of alcohol consumption during pregnancy, 14% of the population sampled said yes. The most commonly written in explanation related to a scare tactic to reduce the chances of FASD; however, these responses nonetheless may be based on assumptions that (a) some alcohol consumption is safe and (b) medical professionals are not providing accurate information. This incredibly important and novel finding suggests possible complications for medical interventions concerning maternal alcohol usage: dissemination of scientifically-based knowledge concerning

deleterious effects of alcohol may be hampered by the general public's

unwillingness to accept this information as accurate and relevant. Additionally

almost 9% of respondents thought that medical professionals underestimated the

dangers of alcohol. This means a noticeable percentage either thought medical

professionals were intentionally deceptive or were not knowledgeable enough to

provide accurate information.

For purposes of this survey tap water was considered safe and unsafe by

relatively similar amounts of respondents. This makes sense and an be heavily

based on the water of the area you live in. Energy drinks and coffee were seen

as unsafe by 91% and 83% of respondents respectively. The use of high

caffeinated drinks poses a potential risk to a developing infant and can increase

heart rate ad increase the chance of miscarriage (Weng et al., 2008). So

although a large percentage of respondents did categorize energy drinks as

unsafe, it may also be an area for educational intervention. Tea, although lower

in caffeine, could pose similar risks depending on the type and amounts

consumed. Additionally, tea can include types that are low or non-caffeinated,

complicating the results of this question. Sixty-one percent of respondents

considered it relatively safe; therefore, special care must be taken to ensure the

risks of caffeinated drinks, including certain teas, are understood.

Medications: The use of medications during pregnancy could pose a potential

risk to mother and baby. Unfortunately, there is a paucity of research delving into

some of the effects of medications during pregnancy. A review from 2001 found

that there is little research into the teratogenicity of 90% of medications approved by FDA between 1980 and 2000 (Andrade et al., 2004). In this survey anti-depressants received the lowest safety score with only 3% stating it as safe and 88% listing it as unsafe. The use of anti-depressants and their safety during pregnancy is still being explored; however, research does suggest a risk of preterm birth, low birth weight, respiratory distress, and neonatal convulsions (Patel et al., 2011). This was followed by Non-prescription cold medications. Aspirin and Ibuprofen all had similar perceptions according to the survey and animal models exploring the effects of analgesics like these suggest a number of developmental malformations (Burdan et al., 2006). Prescription pain medication had the largest percentage of safe responses, with 11% listing them as safe and 20% listing them as somewhat safe. Studies have made links between prescription opioid use and congenital heart defects (Bracken et al., 1986). Due to the increasing use of prescription pain medications, this is an area we must strive to effectively communicate our current scientific understanding while new research is conducted.

Gender differences

Based on the Kruskal-Wallis tests exploring the differences between men and women's responses on the safety of food, beverage and medication items, several items were perceived differently between the two genders. Specifically, men perceived tuna, tap water, energy drinks, coffee, wine, cold medication,

aspirin, ibuprofen and tylenol as significantly safer than the women in our study did (Figure 21). This corroborates additional research showing men rate certain food items as safer compared to women (Burger et al., 1998, Baker et al., 2003) and should be considered when designing educational programs.

Study limitations

Our data includes students surveyed using the undergraduate pool at a southern California University, so the group consisted of college-educated students with the majority between the ages of 18-24 (89%) (Table 4). Thus, differences in socioeconomic status and background are not well represented in this survey. There was also not an even representation of ethnicity, 43% were Asian/Pacific Islander and 34% were Hispanic, with much smaller percentages for White, Middle Eastern and African American (Table 4). Although our ordinal regression analysis did not produce any significant effect for gender/ethnicity as a predictor of responses, the disparity between the represented ethnicities may be limiting these results. This also means that small sample sizes for the less represented groups (White, Middle Eastern and African American) made assessing cross-cultural differences less powerful. The survey was designed to be a relatively short questionnaire aimed at assessing the perception of general dietary habits, it was not meant to be a complete assessment of healthy practices during pregnancy. The survey was limited to the overall perception of safety and did not include the assessment of the amounts per serving that could be considered safe versus unsafe, or take into account exercise, pre-existing health conditions or

additional health effects. This should be considered because each could play a

role in determining the health outcomes of mother and child and may have

played a role in participant's responses to the questionnaire.

Conclusion

Recent work by our lab is exploring ways to ameliorate the effects of PrEE. This

is preliminary work and requires concurrent treatment along with the exposure to

alcohol. Although important in understanding the mechanisms behind alcohol's

teratogenicity, it is a long way from providing treatment for FASD cases.

Treatment in humans is further complicated by the fact that diagnosis of FASD

requires an admission, by the mother, of alcohol consumption, something not

always easy to obtain. At the moment, it is apparent that the most effective

method of reducing the prevalence of conditions such as fetal alcohol spectrum

disorders (FASD) is not by treating the symptoms, but preventing the behavior

that caused it. In order to do this we must educate the public with our most

current conclusions. This study aimed to understand what basic food, beverage

and medication products people perceive as safe or unsafe during pregnancy.

Without previous work on the perception of safe habits during pregnancy, we

cannot conclude whether public opinion's have changed over time as the

scientific communities' conclusions have developed based on continued

research. Our most relevant data is the number of illnesses related to certain

maternal behaviors, such as FASD. Data suggests approximately 10 per 1,000

children (1%) are born with fetal alcohol effects (May et al., 2001). The CDC

estimates births from 2014 at 3,988,076 in the United States; even a small

misconception about the safety of something like alcohol can result in a very

large number of birth defects that could have been prevented. Efforts to educate

the public about the safety of commonly consumed items must continue,

especially as research into the effects of alcohol, canned foods, medications and

high sugar diets moves forward. The current study found that although most

alcohol is perceived as unsafe during pregnancy, wine is still perceived as safer.

Even a small misconception of safety can result in many avoidable birth defects.

Approximately 5% of respondents stated that they would continue to drink

alcohol during the pregnancy. This is disquieting because exposure during the

early phase of pregnancy has the potential to create the most detrimental effects,

as animal studies have suggested that early exposure (first trimester) produces

significant changes and neurological damage (Coles 1994). The survey also

found that medications, especially prescription pain medications, are perceived

as safe by a sizeable percent of the participants. In terms of food items, canned

food, sweet desserts and salmon appear to have larger percentages of

respondents listing them as safe then the data may warrant. Special attention

may be needed for items with hidden teratogens like canned foods and fish. The

most troubling are the rising rates of prescription painkiller use and the perceived

safety of them during pregnancy. As educators we should continue to

communicate our most current understanding of the safest practices for pregnant women.

Additionally, based on an open ended question regarding information from their doctor, it was found that a considerable number of participants thought medical professionals were either deceptive or underestimated the dangers of something like alcohol use during pregnancy. This is something we, as educators, must keep in mind moving forward. It suggests the perception that the medical and scientific field is unsure of the potential dangers of alcohols effect on a developing child. Our goal should be not only communicating our findings as scientists, but to foster trust and confidence in science throughout the community to avoid this type of mistrust.

General conclusions

This dissertation builds on recently published work demonstrating a heritable phenotype caused by prenatal alcohol exposure. Changes measured at birth in the first generation are extended to track the pathology through early development and reproduction.

Using a CD-1 mouse, we designed and studied a model of Fetal Alcohol Spectrum Disorders with the intent of cataloging the generational changes that result from prenatal ethanol consumption during pregnancy through the paternal germline. Specifically, we show how anatomy, neocortical circuitry and behavior are altered in both newborn mice as well as in twenty-day-old mice. The specific

set of connections seen in P0 PrEE mice (F1-F3) represents a unique phenotype that is not observed in control animals of any age. We also detected changes in cortical thickness and volume of select structures in brain at P0. MRI- based studies in children with FASD document a correlation between connectional abnormalities and alterations in region-specific cortical volume (Sowell et al., 2008; Treit et. al, 2014). This suggests that the aberrant connections documented in Chapter 1 may contribute to the cortical thickness changes observed at birth in our model. The fact these projections persist transgenerationally suggests the underlying gene expression may also be perturbed, as deviations to patterned gene expression in cortex is well known to elicit broad changes in cortical connectivity (Huffman et al., 2004).

The fact that the changes documented at birth do not all persist into early development complicates the story. Ideally, changes to behavior that correspond to alterations in anatomy and connectivity would be a simple relationship to conceptualize. However, here we document strong behavioral alterations across generations without all the same changes found at birth being present when behavior is analyzed. This may represent a change in development that the brain is slow or incapable of recovering from or the changes may be at a smaller scale than is detectable by the tools employed here. This would not be unique to alcohol, as prenatal exposure to cocaine can result in permanent changes to neuronal migration and behavior (Standwood et al., 2001; Lidow 1998). The data presented here corroborates long-term human studies. Spohr and Willms report

that children diagnosed with FAS showed consistent behavioral and cognitive retardation despite other neurobiological factors improving (2003). This lends further support to this being a useful model of FASD. Finally, we show interesting results on two behavioral assays that highlight anxiety-like behaviors. The elevated plus maze, a classic test for anxiety-like responses suggested PrEE F1, F2 and F3 mice showed less anxiety by spending increased time in the open arms. The suok test results suggest the opposite, reductions in exploratory behavior and a higher latency to begin the test is generally interpreted as increases in anxiety. This complicates the general interpretation of these two tests and calls into question how we as scientists are assessing a complex psychological phenomenon like anxiety. Further study into the differences discovered from these two tests is necessary to begin exploring the possible difference in anxiety-like behavior, impulsivity or behavioral inhibition that may be at play in these two behavioral assessments.

The persistent, epigenetic, changes caused by PrEE may be the result of altered methylation profiles of select genes responsible for cortical development and axonal targeting (Abbott et al., 2017). The discovery of identifiable changes in brain that persist through reproduction and into the early postnatal period highlights the significant risk of alcohol consumption during pregnancy. A risk that is not only present within the directly exposed generation, but to their offspring as well.

Lastly we examined the perceived safety of common food, beverage and medication items during pregnancy. This highlighted several areas of misconceptions in the public's assessment of safe dietary habits. Foods containing high levels of bisphenol-A, and methyl-mercury were believed to be safe or somewhat safe by a large percentage of participants. Prescription pain-medications (opiates) were also considered safe by a surprisingly large percentage. The perceived safety of alcohol in pregnancy varied based on the type of drink with relatively small percentages suggesting any type was safe or somewhat safe. However, 14-17% listed wine as safe to consume sporadically during certain trimesters. Additionally we highlighted possible areas that mistrust of information from experts may be coming from, as respondents appeared to agree on an uncertainty of information from their medical professional.

In conclusion, this dissertation identifies long-lasting, multi-generational effects of prenatal alcohol exposure on neurobehavioral development at two different ages. In addition it supports the efficacy of this model as a tool for studying the effects of prenatal alcohol exposure. Cessation of alcohol consumption during pregnancy is the most effective method in preventing FASD and understanding whether the public views this as true is an important step in improving our education about the dangers posed by alcohol consumption for the developing infant.

Figures

Figure 1. *Breeding paradigm used to establish a transgenerational model of PrEE*. Alcohol exposure occurs during pregnancy of the first filial generation mice with 25% (v/v) ethanol consumed *ad libitum*. Breeding of second and third generation mice is done by pairing first and second generation males with control females respectively.
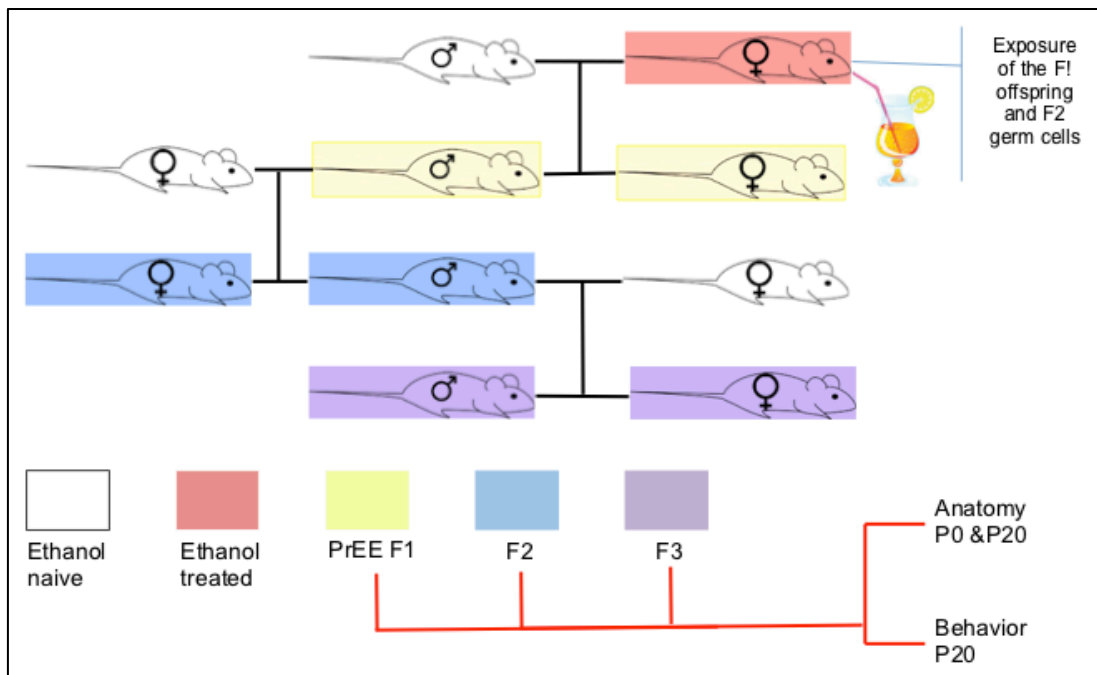
Figure 2. *Measurements in control, F1, F2, and F3 dams*. Average dam food intake (g/day; A) showed no significant differences. Average gestational weight gain in grams (B). F1, F2, and F3 dams gained significantly less weight than control dams over the gestational period. Average litter size (#pups; C). F1, F2, and F3 dams gave birth to significantly smaller litters than control dams. Average dam plasma osmolality on GD 18.5 (mosm/kg; D) showed no significant differences. Average dam blood ethanol content (mg/dL) at GD 9 and 19 (E). F1 dams with EtOH treatment showed significantly higher BEC levels compared to controls. As control, F2, and F3 dams did not receive EtOH treatment, there was no ETHO detected. Data expressed as mean ± S.E.M. * *p<0.05* and **** *p<0.0001*
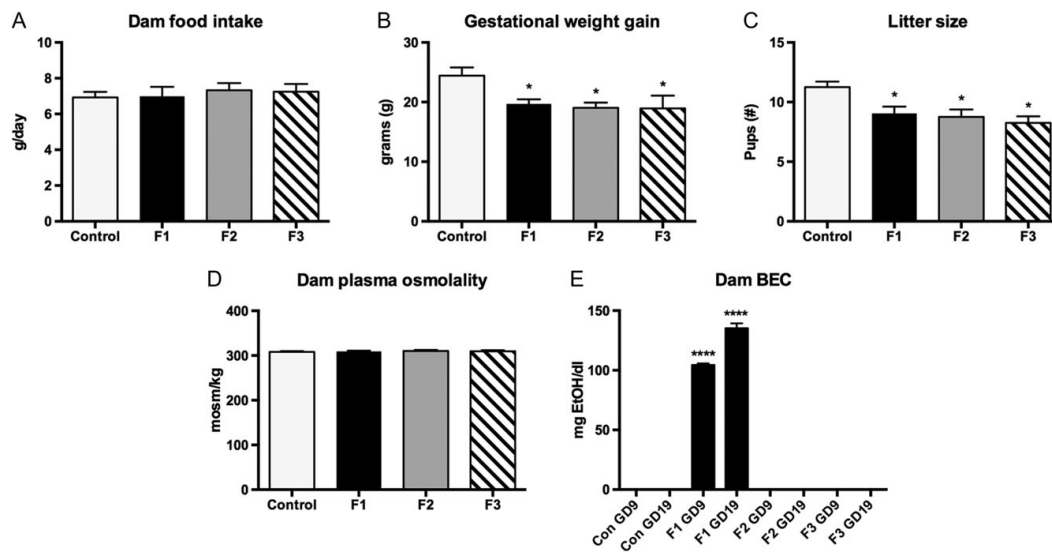
Figure 3. *Body weight, brain weight and cortical length in F1, F2 and F3 mice.* Body weight (A; n=20 all groups), brain weight (B; Control n=8, F1 n=7, F2&F3 n=5) and cortical length (C; all groups n=6) were reduced in all three generations of newborn mice. Data expressed as mean ± S.E.M. * *p<0.05*, ** *p<0.01* and *** *p<0.001*.
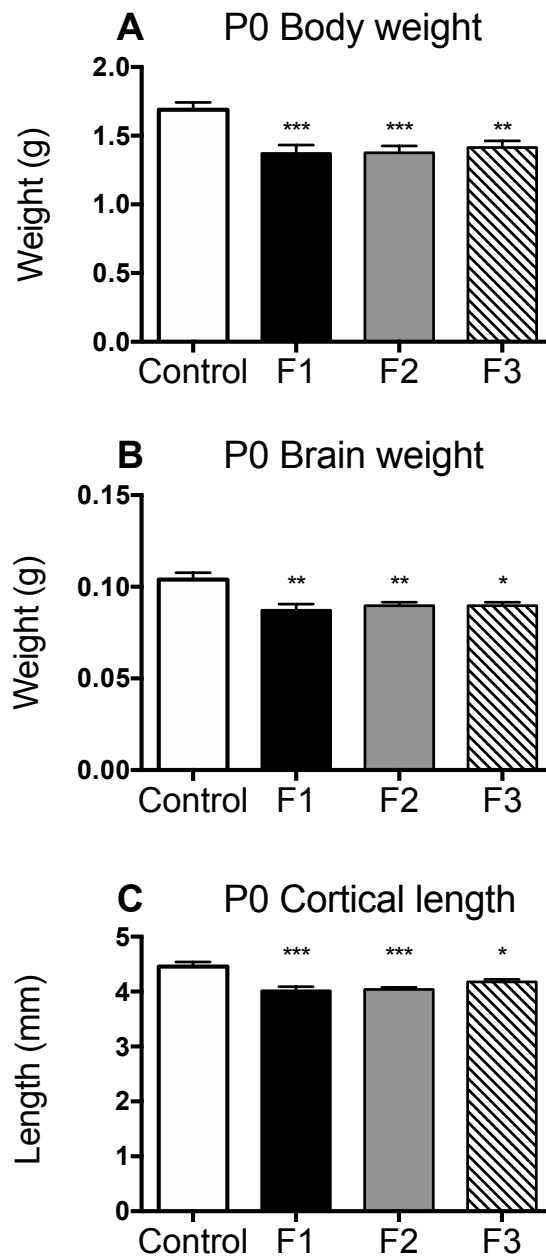
Figure 4. *Dorsal view of newborn whole brains*. Dorsal images of control, F1, F2 and F3 (PrEE) mice brains. PrEE brains are reduced in size when compared to age-matched controls. Scale bar = 2 mm.
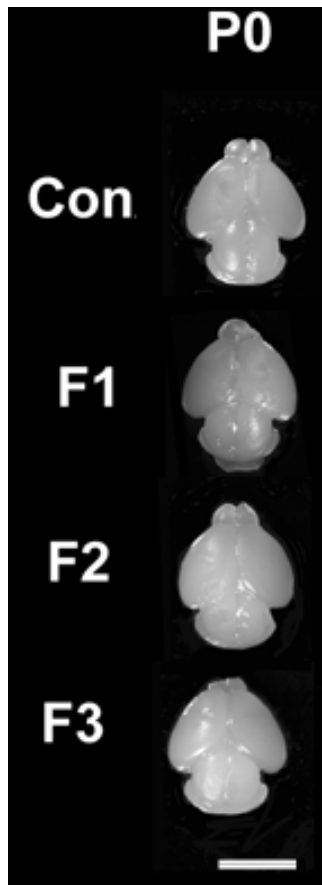
Figure 5. *Development of putative somatosensory and visual INCs in control, F1, F2, and F3 brains at P0*. Rostral to caudal series of 100 μm coronal sections of P0 hemi-spheres following dye placements of DiA (green) in putative somatosensory cortex (A2, B2, C2, D2, asterisks) or DiI (red) in putative visual cortex (A5, B5, C5, D5, asterisks). For all coronal sections, DAPI (blue) was used as a counterstain; arrows indicate ectopic retrogradely labeled cells. Ectopic, red DiI labeled cells from putative visual cortex DPLs are present in rostral cortical regions of F1 (B1–3), F2 (C1–C3), and F3 (D1–D3) brains, but are absent in controls (A1–A3). Ectopic, green DiA labeled cells from putative somatosensory cortex DPLs are present in caudal regions in F1 (B4), F2 (C4), and F3 (D4) brains, but are absent in controls (A4). Images are oriented dorsal (D) up and lateral (L) to the right. Scale bar, 500 μm.
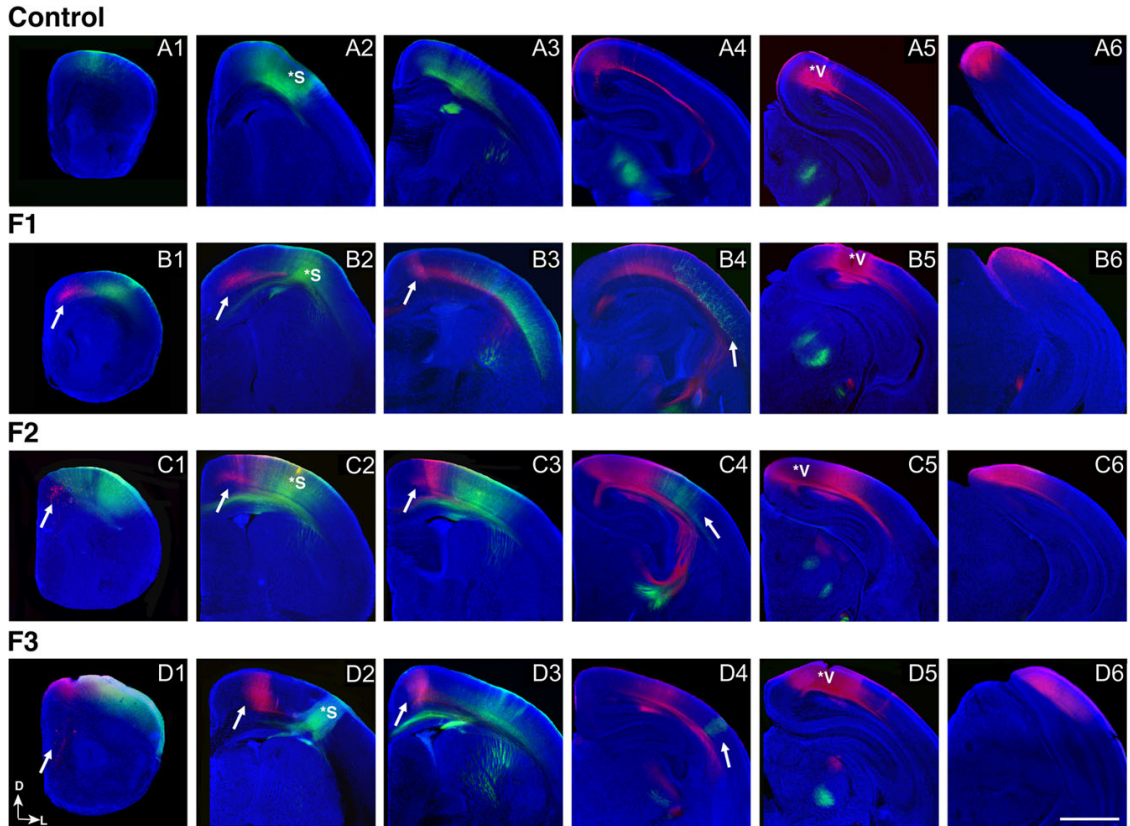
Figure 6. *Average cortical DPL spread and projection zones in control F1, F2, and F3 P0 brains.* (A) Average spread of putative somatosensory cortex (SomCx) DPLs. There were no significant differences in DPL spread across groups (B) Average projection zone of retrogradely labeled cells from putative SomCx DPLs. Somatosensory cortex projection zones in F1 and F2 brains were significantly increased when compared to controls, while F3 showed a trend to significance (p=0.07). (C) Average number of ectopic cells labeled from putative SomCx DPLs. Ectopic, caudal cell labeling from putative SomCx DPLs was significantly increased in F1, F2, and F3 generations when compared to controls. (D) Average spread of putative visual cortex DPL. There were no significant differences in DPL spread across groups (E) Average projection zone of retrogradely labeled cells from putative visual cortex (VisCx) DPLs. Visual cortex projection zones in F1, F2, and F3 brains were significantly increased when compared to controls. (F) Average number of ectopic cells labeled from putative VisCx DPLs. Ectopic, rostral cell labeling from putative VisCx DPLs was significantly increased in F1, F2, and F3 generations when compared to controls. DPL spread and projection zones of labeled cells were taken as a percentage of entire cortical length; the number of ectopic cells labeled from respective DPLs was calculated from cell counts per case. * p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001. Control n=10, F1 n=10, F2 n=9, and F3 n=10. Data expressed as mean ± S.E.M.
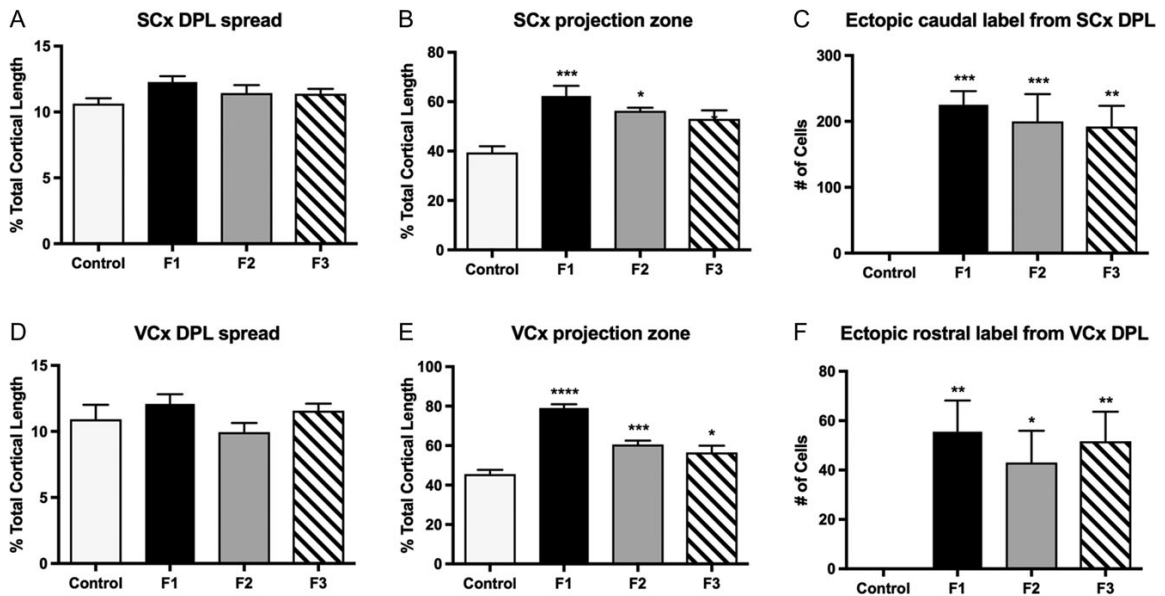
Figure 7. *Cortical thickness of select regions of F1, F2 and F3 mice at P0.*
Coronal sections of representative control (first row), F1 (second row), F2 (third row) and F3 (fourth row) tissue. Arrows indicate area of measure. F1, F2 and F3 mice exhibited significantly thicker frontal (A5; all groups n=15) and somatosensory (C5; all groups n=10) cortices. F1, F2 and F3 mice exhibited a significantly thinner prelimbic cortex (B5; Control n=20, n=10 F1-F3). F1 mice exhibited a significantly thinner auditory cortex (D5; all groups n=10) and thicker visual cortex (E5, all groups n=10). No significant variation was detected in visual and auditory cortices of F2 and F3 animals (D5 & E5 respectively). Data is expressed as mean percent of control ± S.E.M. Images oriented dorsal up (D) and lateral to the right (L). Scale bar 500 µm. * *p<0.05*, and ** *p<0.01*.

Figure 8. *Subcortical anatomy of F1, F2 and F3 mice at P0.* Coronal sections of representative control (first row), F1 (second row), F2 (third row) and F3 (fourth row) tissue. Arrows/outlines indicate area of measure, volumetric measures were take from serial sections. F1, F2 and F3 mice displayed a significantly thinner corpus callosum (A5; Control n=13, F1-F3 n=11). F1 animals exhibited a significantly thinner CA3 (B5, all groups n=9). No significant difference was exhibited by F1, F2 or F3 mice in volumetric measures of the dLGN (C5; all groups n=10), MGN (D5; all groups n=5), and VP (E5; all groups n=7) nucleus of the thalamus at P0. Data is expressed as mean percent of control ± S.E.M. Images oriented dorsal up (D) and lateral to the right (L). Scale bar 500 µm. * $p<0.05$ and ** $p<0.01$.

Figure 9. *Volumetric measures of Amygdala and basal ganglia in P0 control F1, F2, and F3 brains.* Coronal sections of representative control (first row), F1 (second row), F2 (third row) and F3 (fourth row) tissue. Outlines indicate area of measure, volumetric measures were take from serial sections. F1, F2 and F3 mice displayed a significantly smaller amygdala (A5; all groups n=7). F1 animals exhibited a significantly smaller basal ganglia (B5; Control & F1 n=17). F2 & F3 brains showed no significant difference in basal ganglia size (B5; F2 and F3 n=10). Data is expressed as mean percent of control ± S.E.M. Images oriented dorsal up (D) and lateral to the right (L). Scale bar 500 μm. * $p<0.05$ and ** $p<0.01$.
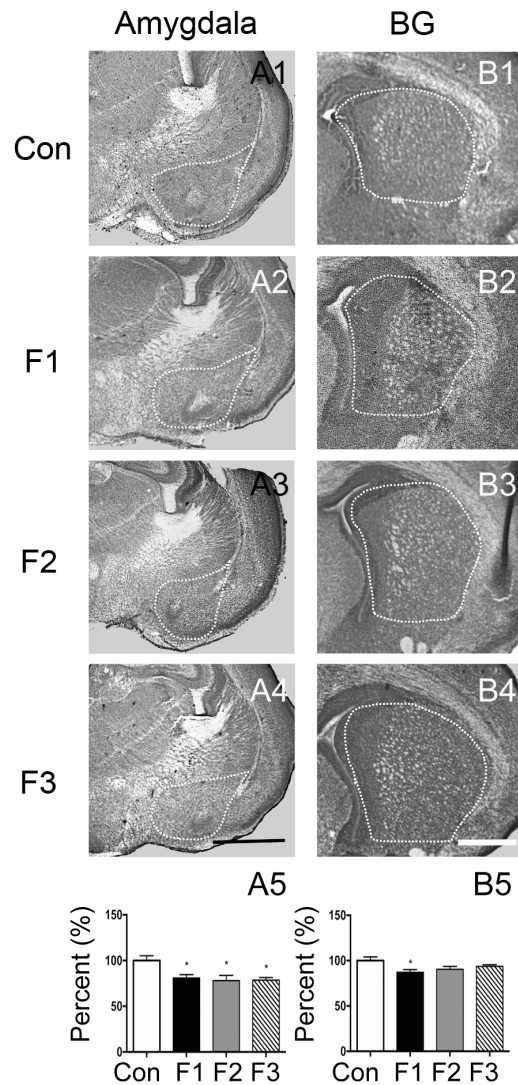
Figure 10. *Body weight, brain weight and cortical length were not different at P20 in F2 and F3 mice.* Body weight (A; Control n=21, F1 n=17, F2 n=34, F3 n=29), brain weight (B; all groups n=7) and cortical length (C; all groups n=6) were reduced in F1 twenty-day-old mice. At P20 F2 and F3 body weight (A;), brain weight (B) and cortical length were not significantly different (C). F1, F2 and F3 brains are reduced in length when compared to age-matched controls at P0.Data expressed as mean ± S.E.M. ** *p<0.01, ***p<0.001*.
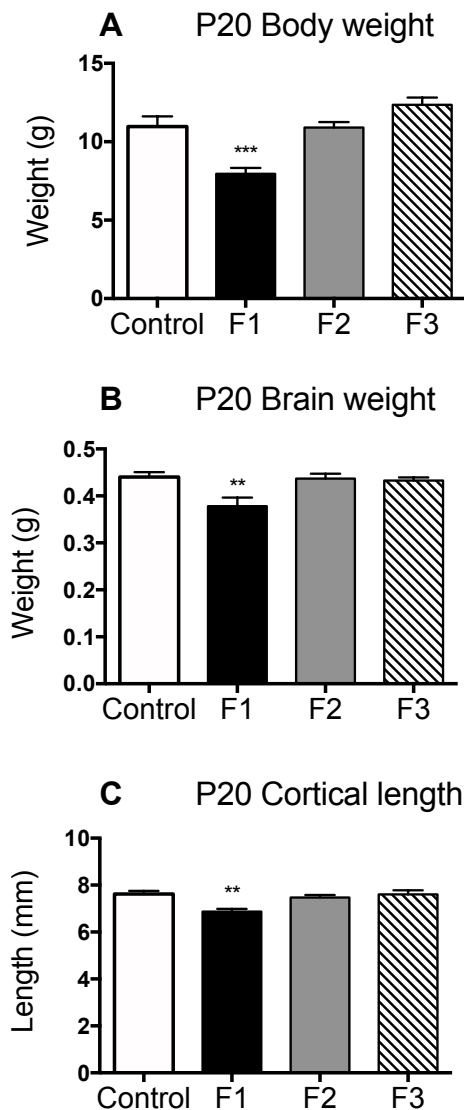
Figure 11. *Dorsal view of twenty-day-old whole brains.* Dorsal images of control, F1, F2 and F3 mice brains. PrEE brains are reduced in size when compared to age-matched controls. F1 brains were significantly smaller compared to control cases. Scale bar = 2 mm.
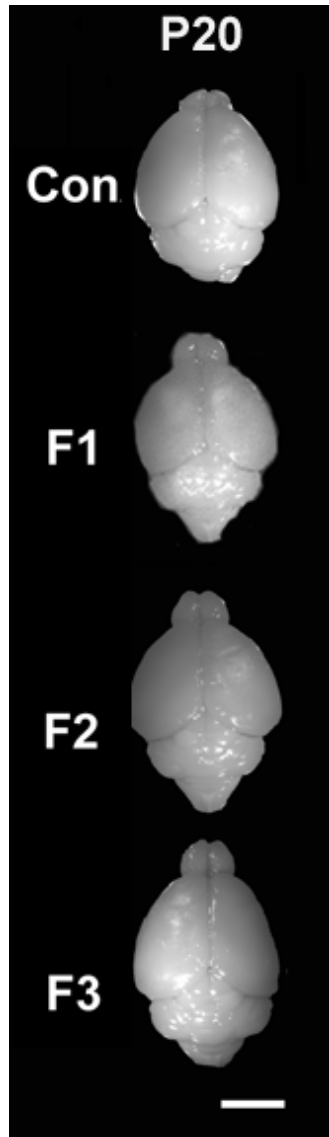
Figure 12. *Somatosensory and visual INCs in control, F1, F2 and F3 FASD brains at P20.* PrEE does not alter INCs in P20 F1, F2 or F3 brains. Rostral to caudal series of 100 um coronal sections of P20 hemispheres following DiA (green) or DiI (red) crystal placements in putative somatosensory (starred C1-C4) and putative visual cortex (starred, E1-E4) of control, F1, F2 and F3 mouse brains. Sections were counterstained with DAPI. In both control and F1-F3 brains, retrogradely labeled cells from a DPL in the somatosensory cortex (C1-C4) are seen rostral and caudal relative to the DPL (control A1-D1, F1 A2-D2, F2 A3-D3, F3 A4-D4). A DPL in visual cortex results in labeled cells rostral and caudal (control C1-F1, F1 C2-F2, F2 C3-F3, F3 C4-F4) to the DPL, with no visible ectopically labeled cells. Images oriented dorsal (D) up and lateral (L) to the right. Scale bar 500 μm.
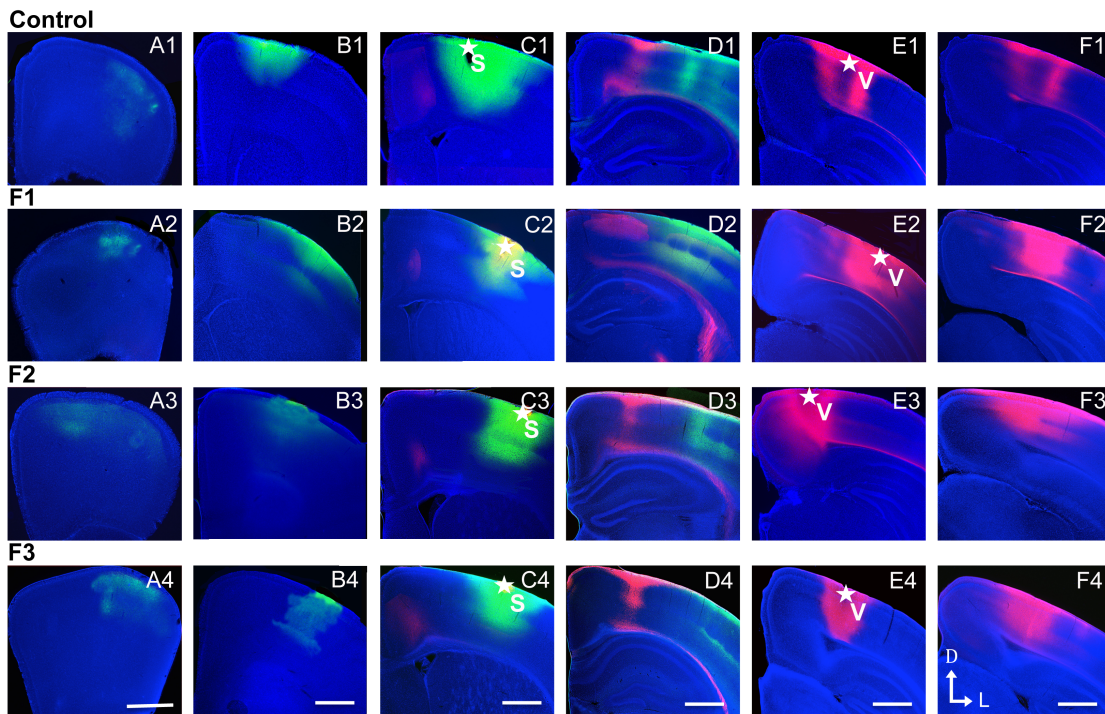
Figure 13. *Cortical thickness measures in control, F1, F2 and F3 mice at P20.* Coronal sections of representative control (first row), F1 (second row), F2 (third row) and F3 (fourth row) tissue. Arrows indicate area of measure. F1, F2 and F3 mice exhibited significantly thicker frontal (A5; n=8 all groups) cortex. F1 mice exhibited significantly thicker prelimbic (B; Control & F1 n=9), somatosensory (C; Control n=8, F1 n=10) and auditory cortices (D5; Control & F1 n=8). F2 and F3 mice did not exhibit a significantly difference in prelimbic (B; F2&F3 n=8), somatosensory (C; p>0.05; F2&F3 n=8) or auditory cortex thickness (D5; F2&F3 n=8). F1, F2 and F3 mice showed no significant difference in visual cortex thickness (E; Control, F2&F3 n=8, F1 n=10). Data is expressed as mean percent of control ± S.E.M. Images oriented dorsal up (D) and lateral to the right (L). Scale bar 500 μm. * $p<0.05$, and ** $p<0.01$.
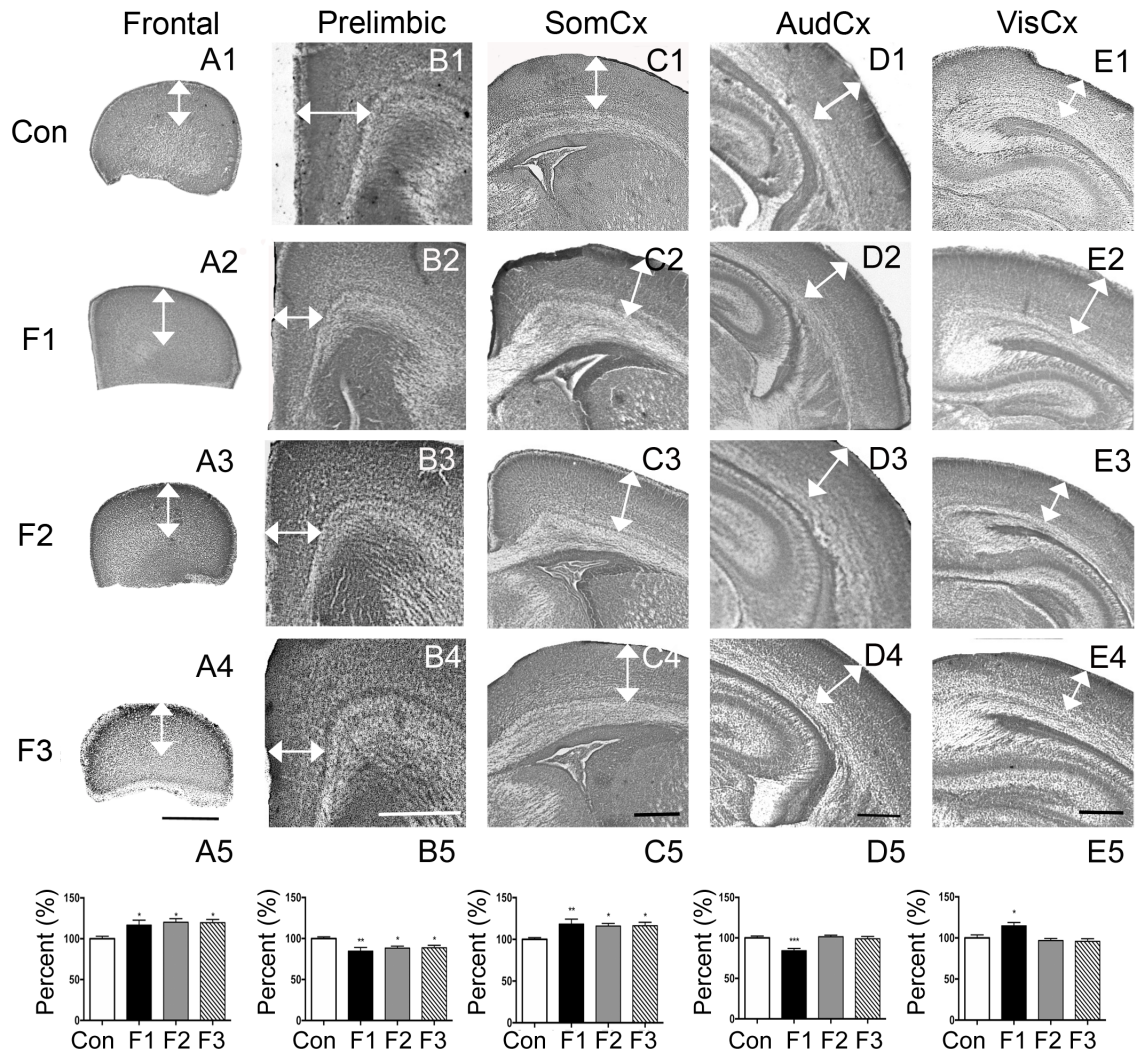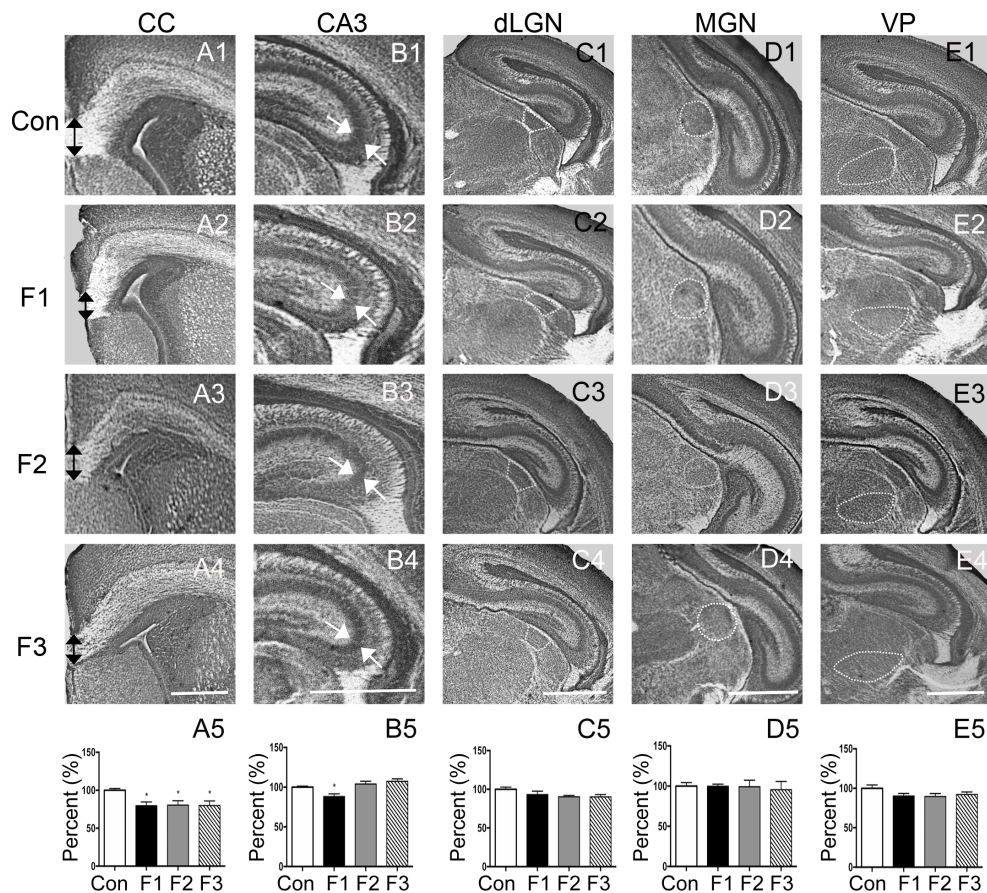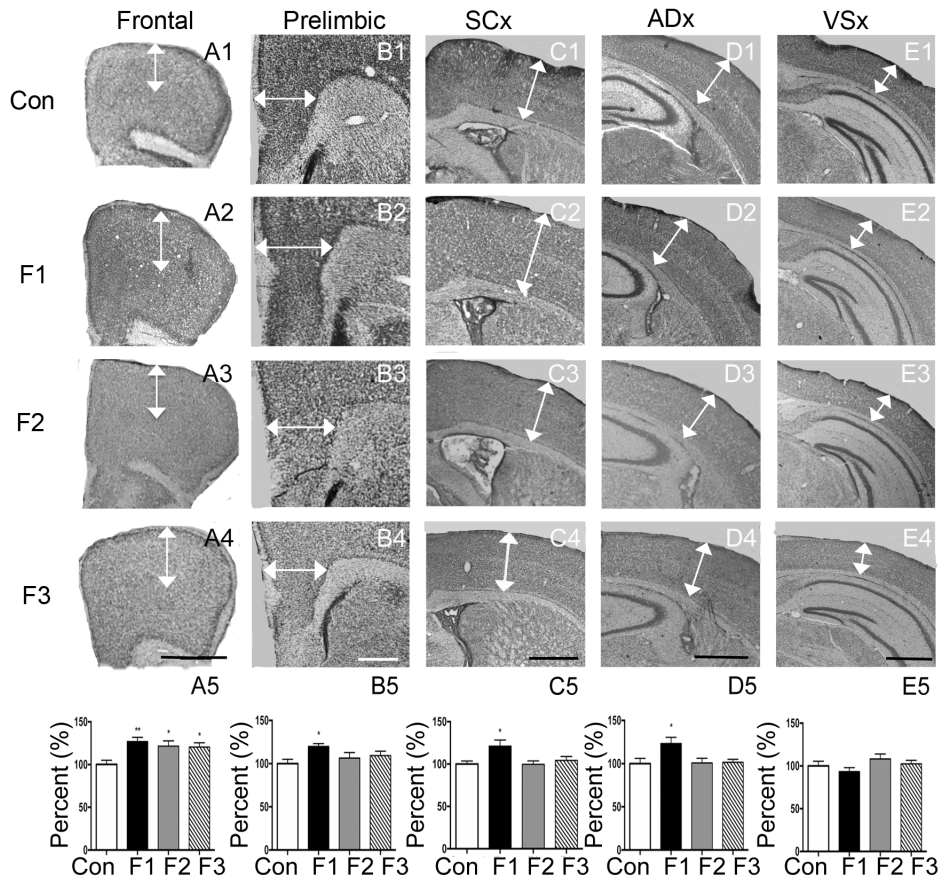
Figure 14. *Subcortical anatomy of F1, F2 and F3 mice at P20.* Coronal sections of representative control (first row), F1 (second row), F2 (third row) and F3 (fourth row) tissue. Arrows/outlines indicate area of measure, volumetric measures were take from serial sections. F1 and F2 mice displayed a significantly thinner corpus callosum (A5; control, F1&F2 n=9). F3 mice showed no significant difference in thickness measures of the corpus callous (A; F3 n=9). F1 animals exhibited a significantly thicker CA3 (B5; Control n=10, F1 n=9) and thinner dLGN (B5; Control&F1 n=8). No significant difference was exhibited by F2 or F3 mice in volumetric measures of the CA3 (B; F2&F3 n=10) region of the hippocampus. No significant difference was exhibited by F1, F2 or F3 mice in volumetric measures of the MGN (D5; n=5 all groups) and VP (E5; n=6 all groups) nucleus of the thalamus at P20. Data is expressed as mean percent of control ± S.E.M. Images oriented dorsal up (D) and lateral to the right (L). Scale bar 500 µm. * *p<0.05*.


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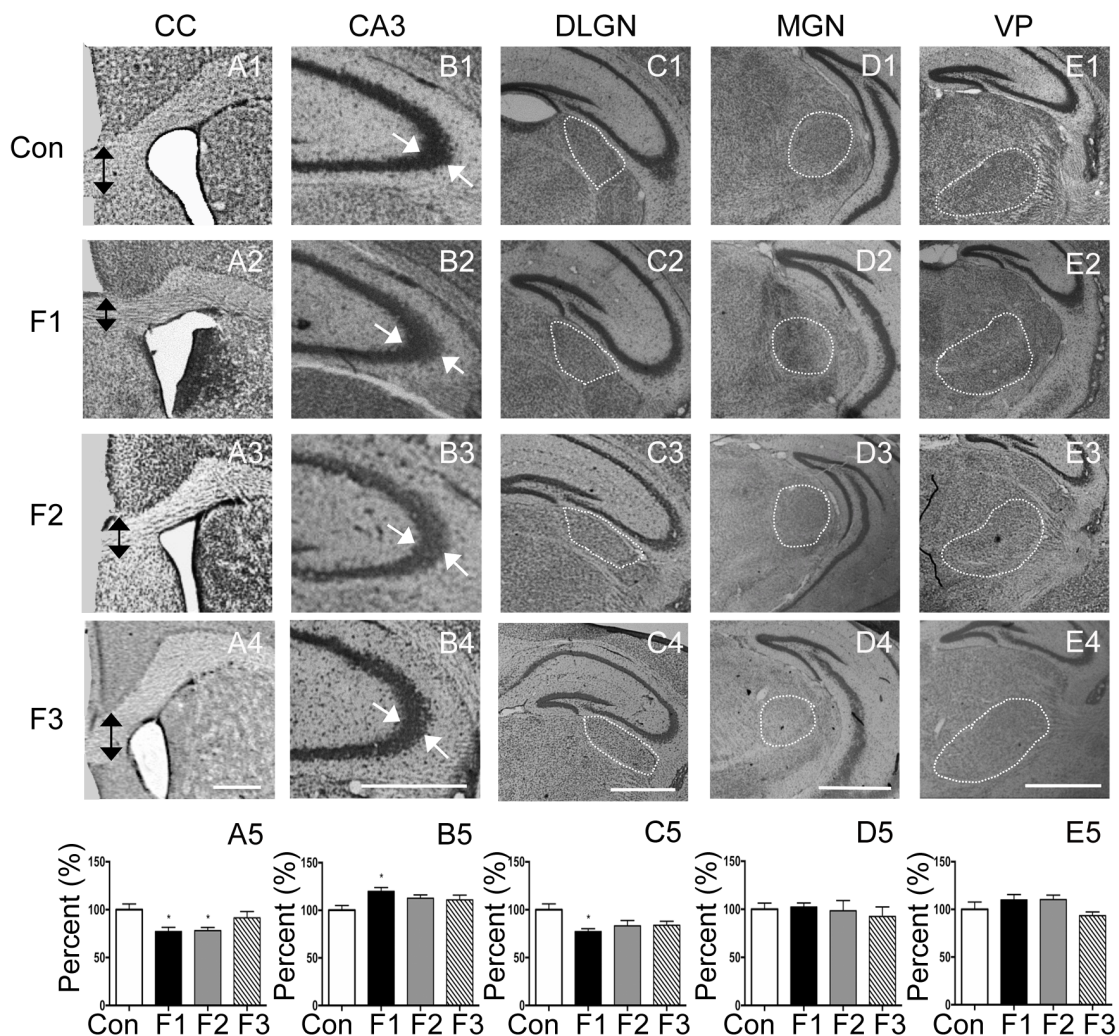
Figure 15. *Volumetric measures of Amygdala and basal ganglia in P20 control F1, F2, and F3 brains.* Coronal sections of representative control (first row), F1 (second row), F2 (third row) and F3 (fourth row) tissue. Outlines indicate area of measure, volumetric measures were take from serial sections. F1, F2 and F3 mice did not display a significant difference in volumetric measures of the amygdala (A5; Control, F1&F3 n=6, F2 n=5) or basal ganglia (A5; Control, F2 n=6, F1&F2 n=7). Data is expressed as mean percent of control ± S.E.M. Images oriented dorsal up (D) and lateral to the right (L). Scale bar 500 µm.
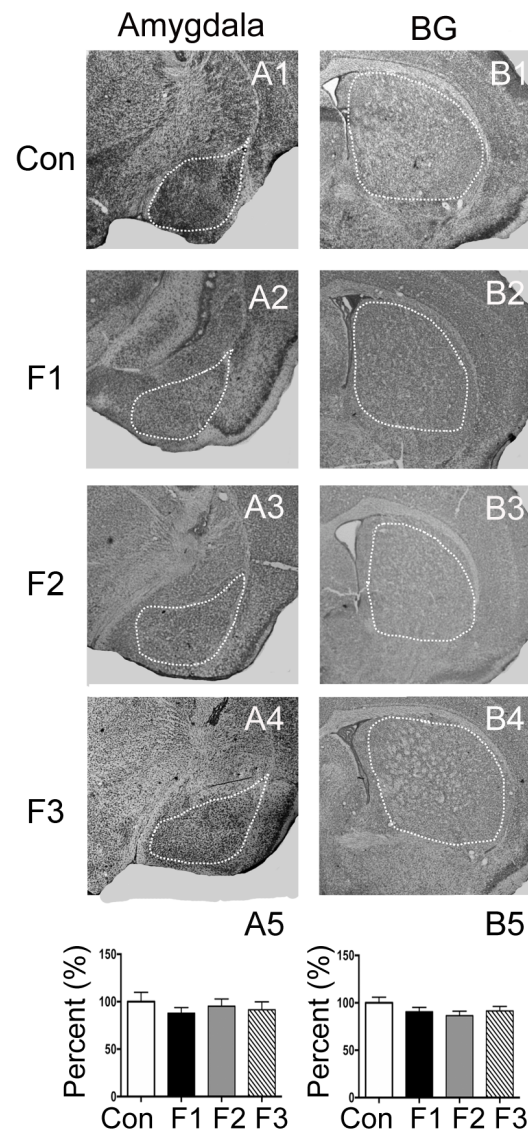
Figure 16. *The Suok bar test and three-chambered sociability test.* F1, F2 and F3 mice exhibited increased anxiety, reduced coordination and reduced social interactions**.** The Suok test behavioral assay performed on mice at twenty days old. Significant group differences were seen in the F1 & F2 generation for latency to leave the center (A), no difference was observed for F3 animals (A). Rearing/grooming showed altered behavior in the F1 & F3 generation (B), F2 animals showed no difference. Directed exploration showed altered behavior in all three generations of mice (C). F1, F2 and F3 mice made significantly more missteps (D). F1, F2 and F3 mice also fell significantly more times than controls (E). During the three-chambered sociability test (F) control mice spent significantly more time with the novel mouse then in the empty chamber during the ten-minute testing phase. F1, F2 and F3 mice did not spend more time with the novel mouse versus time spent in the empty chamber as seen in the smaller ratio (F), suggesting lowered social interaction. Data expressed as mean ± S.E.M. * *p<0.05,* ** *p<0.01,* *** *p<0.001, and* **** *p<0.0001*. Suok: Control n=24, F1 n=19, F2 n=35, F3 n=22; Sociability chamber: Control n=8, F1 n=6, F2 n=7, F3 n=7.
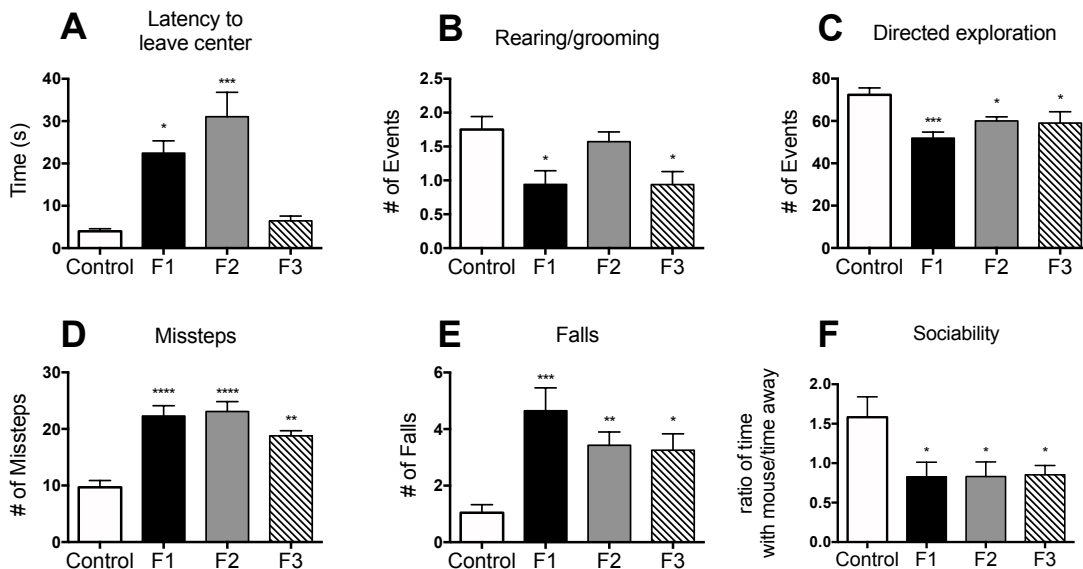
Figure 17. *Rotarod, adhesive removal test, elevated plus maze and forced swim test on P20 control F1, F2, and F3 mice.* Significant group differences were found in motor coordination and learning, with F1, F2 & F3 animals having poor motor coordination and motor learning deficits compared to controls during the rotarod assessment (A; all groups n=9). Significant differences in trial performances were seen in Trial 1 (A) and Trial 2 (A). Significant differences in sensorimotor integration were detected in F1, F2 & F3 animals documented by the increased latencies to detect and remove an adhesive in the adhesive removal test (B; all groups n=11). Significant differences were exhibited in Trial 1 (B) and Trial 2 (B). F1, F2 and F3 animals also displayed anxiolytic-like behaviors, evident in experimental animals spending an increased time in open arms (C; all groups n=8) and a shorter time in closed arms (data not shown) when compared to controls on the elevated plus maze. F1, F2 and F3 animals exhibited significant depressive-like behavior relative to controls during the forced swim test (D; all groups n=8). * $p<0.05$, ** $p<0.01$, *** $p<0.001$, and **** $p<0.0001$.
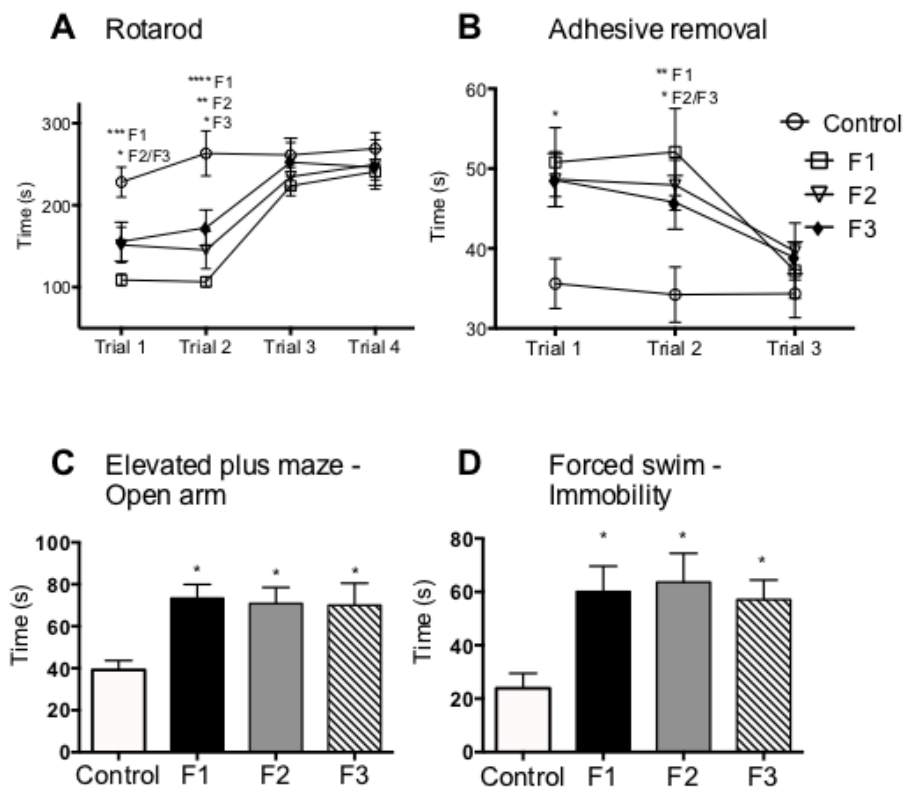
Figure 18. *General perception of food safety during pregnancy.* Graphical depiction of the general perception of relatively safe (combined somewhat safe and safe, white bars) versus relatively unsafe (combined somewhat unsafe and unsafe, black bars) and no opinion (grey bars) for each food item.
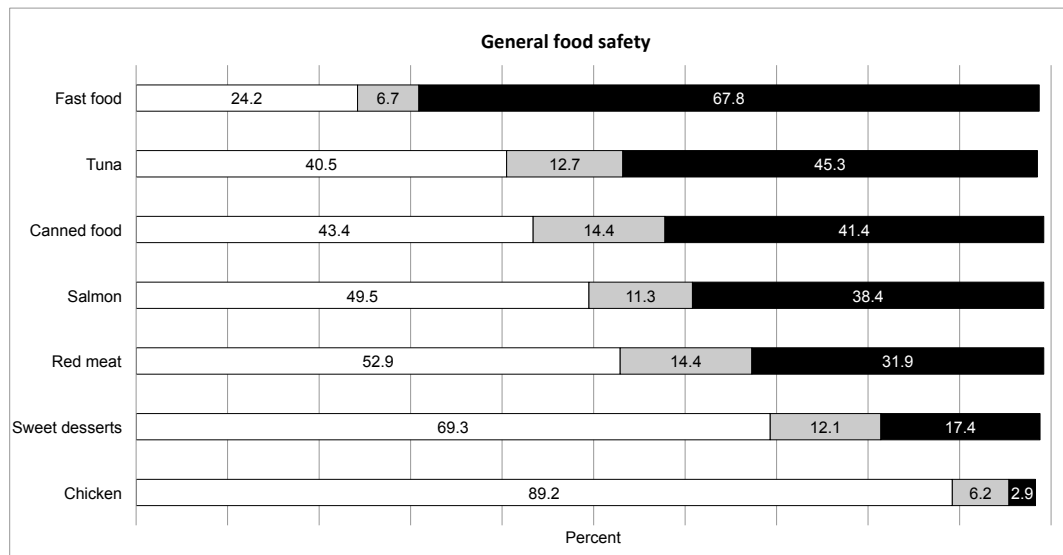
Figure 19. *General perception of beverage safety during pregnancy.* Graphical depiction o**f** the general perception of relatively safe (**c**ombined somewhat safe and safe, white bars) versus relatively unsafe (combined somewhat unsafe and unsafe, black bars) and no opinion (grey bars) for each beverage type.
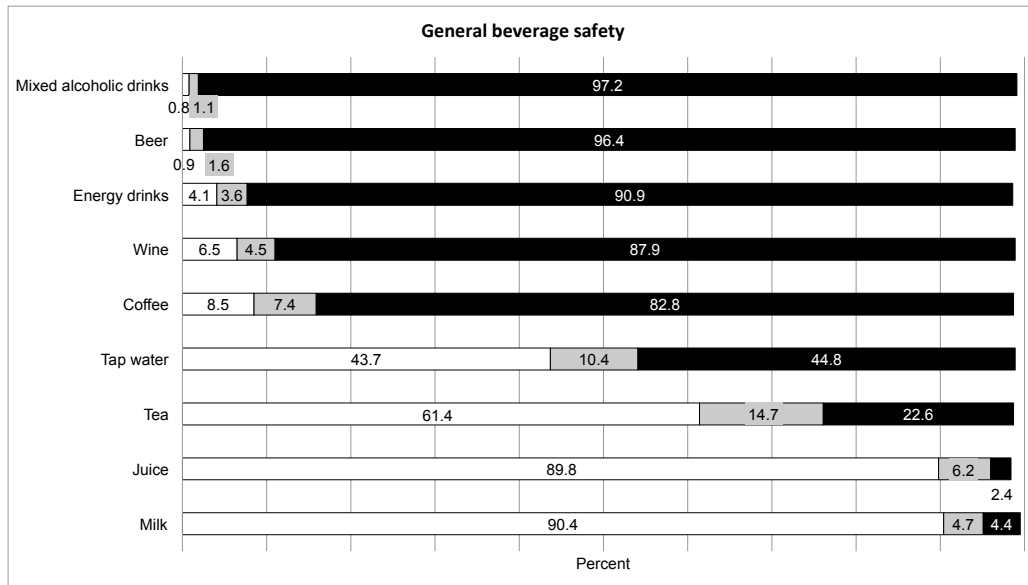
Figure 20. *General perception of medication safety during pregnancy.* Graphical depiction of the general perception of relatively safe (combined somewhat safe and safe, white bars) versus relatively unsafe (combined somewhat unsafe and unsafe, black bars) and no opinion (grey bars) for each medication.
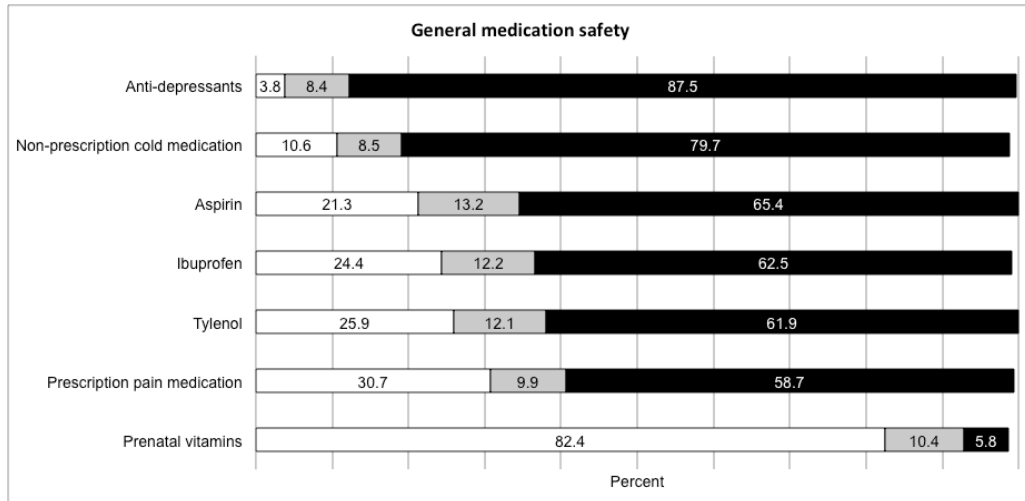
Figure 21. *Gender differences in the perception of safe food, drink and medications.* Percent responses for relatively safe (safe and somewhat safe) based on the perception of tuna, tap water, energy drinks, wine, coffee, cold medication, Ibuprofen and aspirin. Items included are based on non-parametric Kruskal-Wallis tests. In general men reported each item as being safer than women did in this survey.
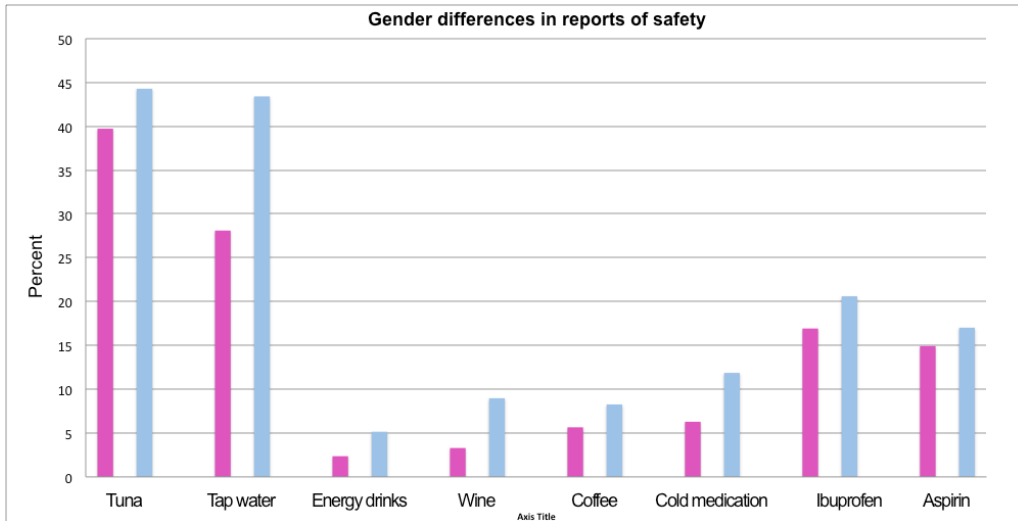
Tables

Table 1. *Abbreviations.*

AudCx	Primary auditory cortex
BNPWS	Belief about the nutrition of pregnant women survey
BG	Basal ganglia
BPA	Bisphenol A
CA3	Cornu ammonis region 3, hippocampus
CC	Corpus callosum
dLGN	Dorsal lateral geniculate nucleus
FAS	Fetal alcohol syndrome
FASD	Fetal alcohol spectrum disorders
INCs	Intraneocortical connections
MeHg	Methyl mercury
MGN	Medical geniculate nucleus
NAS	Neonatal abstinence syndrome
P	Postnatal day
PCB	Polychlorinated biphenyls
PrEE	Prenatal ethanol exposure
SomCx	Primary somatosensory cortex
VP	Ventral-posterior nucleus
VisCx	Primary visual cortex

Table 2. *Cell packing density of select cortical areas at P0.* No differences were observed in select cortical areas at P0

	Frontal				Prelimbic				SCx			
	Con	F1	F2	F3	Con	F1	F2	F3	Con	F1	F2	F3
P0	101.7±2.5	103.5±4.2	111.1±2.2	102.8±4.9	98.6±2.5	83.7±2.0	108.0±2.7	99.6±5.8	105.6±2.4	109.1±3.5	104.9±2.7	106.2±1.7

	VCx				ACx			
	Con	F1	F2	F3	Con	F1	F2	F3
P0	102.5±1.6	104.4±8.1	108.4±2.0	105.7±2.2	106.3±4.2	1.5.8±1.3	100.2±3.3	106.3±4.3

Table 3. *Cell packing density of select cortical areas at P20.* No differences were observed in select cortical areas at P20.

	Frontal				Prelimbic				SCx			
	Con	F1	F2	F3	Con	F1	F2	F3	Con	F1	F2	F3
P20	88±4.6	94.3±5.5	97.2±2.1	98.1±1.8	87.1±4.7	93.5±7.2	95.1±5.9	97.8±1.1	91.0±3.8	94.5±2.2	100.3±4.1	97.0±2.1

	VCx				ACx			
	Con	F1	F2	F3	Con	F1	F2	F3
P20	86.0±4.3	89.4±7.2	92.2±4.6	95.3±1.3	94.3±6.8	90.1±3.5	94.4±9.2	92.4±3.8

Table 4. *Demographics of survey participants.* Sample characteristics of survey participants including gender, age, college year, ethnicity and childbirth history.

Sex	# of Respondents (%)	Ethnicity	# of Respondents (%)
female	440 (68)	African-American	32 (5)
male	205 (32)	Asian/Pacific Islander	276 (43)
Age		Hispanic	222 (34)
18-24	574 (89)	Middle Eastern	30 (5)
21-25	60 (9)	White	83 (13)
26-29	5 (.8)	Already had children	
30-35	1 (.2)	no	632 (98)
40+	1 (.2)	yes	9 (2)
Year in college		Currently pregnant	
1st	227 (35)	no	644 (99.8)
2nd	160 (25)	yes	1 (.2)
3rd	178 (28)		
4th	67 (10)	Total N=645	
5th+	10 (2)		

Table 5. *Food perception counts of survey participants*

		No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
				Count		
Red meat	Female	58	113	122	113	31
	Male	35	47	59	44	17
	African-American N=32	4	8	11	7	2
	Asian/Pacific Islander N=276	47	57	65	77	30
	Hispanic N=219	23	59	75	50	12
	Middle Eastern N=30	9	7	6	6	2
	White N=83	10	29	24	18	2
Chicken	Female	23	280	116	9	3
	Male	17	122	56	4	3
	African-American N=29	2	22	5	0	0
	Asian/Pacific Islander N=275	24	154	82	11	4
	Hispanic N=220	10	153	54	2	1
	Middle Eastern N=30	3	13	14	0	0
	White N=80	1	61	17	0	1
Tuna	Female	53	74	98	96	112
	Male	29	48	41	49	34
	African-American N=30	6	5	4	7	8
	Asian/Pacific Islander N=273	42	55	57	61	58
	Hispanic N=220	21	42	60	44	53
	Middle Eastern N=30	5	2	3	10	10
	White N=82	8	18	15	23	18
Salmon	Female	44	103	113	74	102
	Male	29	47	56	45	26
	African-American N=31	5	5	5	10	6
	Asian/Pacific Islander N=277	32	69	82	42	52
	Hispanic N=220	25	50	55	42	48
	Middle Eastern N=30	4	5	5	7	9
	White N=82	7	21	22	18	14
Canned food	Female	55	73	125	130	53
	Male	38	22	60	60	23
	African-American N=31	4	4	8	15	0
	Asian/Pacific Islander N=275	49	29	66	87	44
	Hispanic N=222	26	37	73	65	21
	Middle Eastern N=30	5	2	7	9	7
	White N=82	9	23	31	15	4
Sweet desserts	Female	46	164	149	73	5
	Male	32	57	76	28	6
	African-American N=32	4	10	16	2	0
	Asian/Pacific Islander N=272	43	94	93	37	5
	Hispanic N=220	16	75	81	44	4
	Middle Eastern N=30	6	10	10	4	0
	White N=83	9	33	25	14	2
Fast food	Female	26	25	86	196	99
	Male	17	8	37	82	59
	African-American N=30	3	1	3	15	8
	Asian/Pacific Islander N=274	24	14	48	109	79
	Hispanic N=220	6	8	53	108	45
	Middle Eastern N=29	5	3	3	12	6
	White N=83	5	7	16	35	20
Fresh fruit	Female	4	415	16	1	1
	Male	7	184	10	1	1
	African-American N=31	0	30	1	0	0
	Asian/Pacific Islander N=277	6	253	16	1	1
	Hispanic N=220	4	208	7	0	1
	Middle Eastern N=30	1	28	0	1	0
	White N=83	0	81	2	0	0

Table 6. *Beverage perception counts of survey participants*

		No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
				Count		
Tap Water	Female	44	81	98	121	90
	Male	23	50	52	50	28
	African-American N=32	2	3	7	10	10
	Asian/Pacific Islander N=277	33	58	63	75	48
	Hispanic N=218	22	32	55	62	47
	Middle Eastern N=29	4	7	8	7	3
	White N=82	6	31	18	17	10
Milk	Female	21	309	87	16	3
	Male	9	132	54	9	0
	African-American N=32	1	24	5	2	0
	Asian/Pacific Islander N=278	18	184	61	13	2
	Hispanic N=218	7	161	47	3	0
	Middle Eastern N=30	2	22	5	1	0
	White N=83	2	51	23	6	1
Juices	Female	31	279	118	6	1
	Male	9	125	56	8	0
	African-American N=32	4	16	11	1	0
	Asian/Pacific Islander N=272	21	162	84	5	0
	Hispanic N=218	8	149	54	6	1
	Middle Eastern N=29	3	14	11	1	0
	White N=83	4	64	14	1	0
Colas	Female	42	15	59	182	139
	Male	24	4	31	90	50
	African-American N=31	5	0	4	11	11
	Asian/Pacific Islander N=276	33	9	31	122	81
	Hispanic N=217	19	5	33	103	57
	Middle Eastern N=30	3	1	3	11	12
	White N=83	6	4	20	25	28
Energy drinks	Female	2	1	2	8	56
	Male	5	2	3	13	64
	African-American N=32	0	3	3	3	91
	Asian/Pacific Islander N=273	4	2	4	17	73
	Hispanic N=220	2	1	2	11	84
	Middle Eastern N=30	10	0	3	10	77
	White N=81	5	0	2	7	85
Coffee - regular	Female	31	7	29	143	225
	Male	17	6	13	84	81
	African-American N=29	3	1	1	4	20
	Asian/Pacific Islander N=275	20	5	12	109	129
	Hispanic N=221	11	5	17	76	112
	Middle Eastern N=30	5	0	1	9	15
	White N=82	9	2	11	29	31
Black or green tea	Female	59	129	141	66	38
	Male	36	63	63	31	11
	African-American N=29	4	11	9	2	3
	Asian/Pacific Islander N=276	45	80	91	39	21
	Hispanic N=220	28	64	77	37	14
	Middle Eastern N=30	6	7	8	6	3
	White N=82	12	30	19	13	8
Beer	Female	5	0	4	10	416
	Male	5	2	0	7	188
	African-American N=32	0	0	0	0	32
	Asian/Pacific Islander N=274	5	1	1	8	259
	Hispanic N=220	4	1	1	1	213
	Middle Eastern N=29	0	0	0	2	27
	White N=83	1	0	2	6	74
Wine	Female	16	2	19	58	340
	Male	13	5	16	36	132
	African-American N=32	3	0	2	5	22
	Asian/Pacific Islander N=274	14	2	17	47	194
	Hispanic N=219	6	3	11	22	177
	Middle Eastern N=30	3	1	1	3	22
	White N=83	3	1	4	17	58
Alcoholic mixed drinks	Female	3	0	2	6	423
	Male	4	2	1	3	194
	African-American N=32	0	0	0	1	31
	Asian/Pacific Islander N=276	5	1	2	4	264
	Hispanic N=219	2	1	1	2	213
	Middle Eastern N=30	0	0	0	0	30
	White N=82	0	0	0	2	80

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Table 7. *Medication perception counts of survey participants*

		No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
				Count		
Cold medication - non prescription N=637	Female	31	6	34	155	206
	Male	24	4	24	77	75
	African-American N=32	3	0	4	13	12
	Asian/Pacific Islander N=275	30	4	23	94	124
	Hispanic N=221	12	5	15	85	104
	Middle Eastern N=29	5	0	1	9	14
	White N=80	5	1	15	32	27
Tylenol N=644	Female	45	23	89	157	125
	Male	33	7	47	72	45
	African-American N=32	3	2	6	6	15
	Asian/Pacific Islander N=278	41	8	50	101	78
	Hispanic N=221	23	12	51	85	50
	Middle Eastern N=30	6	2	6	7	9
	White N=83	5	6	24	30	18
Ibuprofen N=639	Female	45	17	91	142	141
	Male	34	8	40	71	49
	African-American N=31	4	1	4	8	14
	Asian/Pacific Islander N=277	38	11	47	93	88
	Hispanic N=219	23	9	45	81	61
	Middle Eastern N=30	7	1	7	5	10
	White N=82	7	3	29	26	17
Aspirin N=644	Female	49	14	82	129	166
	Male	36	7	33	68	59
	African-American N=32	4	1	5	5	17
	Asian/Pacific Islander N=278	39	8	49	89	93
	Hispanic N=222	26	8	39	66	83
	Middle Eastern N=30	6	1	3	9	11
	White N=82	10	3	20	28	21
Prenatal vitamins N=635	Female	36	305	69	14	10
	Male	31	102	54	7	6
	African-American N=32	1	23	5	1	2
	Asian/Pacific Islander N=275	43	145	67	12	8
	Hispanic N=217	16	155	37	5	4
	Middle Eastern N=30	3	19	7	1	0
	White N=81	4	66	7	2	2
Anti-depressants N=642	Female	30	1	13	100	293
	Male	24	2	8	45	125
	African-American N=32	1	0	2	5	24
	Asian/Pacific Islander N=277	25	1	7	63	181
	Hispanic N=220	15	1	9	41	154
	Middle Eastern N=30	2	0	0	10	18
	White N=83	11	1	3	26	42
Pain medication - prescription N=641	Female	39	53	85	103	157
	Male	25	18	42	50	68
	African-American N=32	4	4	4	6	14
	Asian/Pacific Islander N=277	35	28	52	66	96
	Hispanic N=221	15	31	53	49	73
	Middle Eastern N=29	4	3	8	8	6
	White N=82	6	5	10	24	37

Table 8. *Frequency data for perception of food*

Red meat

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	14.4	24.8	28.1	24.5	7.4

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	33.5	38	38.9
1-3 times per week	37.1	37.4	29.6
4-6 times per week	13.2	8.8	9.8
7+ times per week	2.5	1.4	2.6
Never Safe	13.5	12.9	17.5

Salmon

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	11.3	23.3	26.2	18.4	20

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	30.7	34	32.9
1-3 times per week	28.2	25.6	26.4
4-6 times per week	12.6	10.7	9.8
7+ times per week	4.8	4.7	4.8
Never Safe	23.3	24	25.3

Fast food

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	6.7	5.1	19.1	43.3	24.5

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	52.1	55.5	54.7
1-3 times per week	19.7	14.9	14.4
4-6 times per week	3.9	2.3	1.7
7+ times per week	1.2	0.6	0.6
Never Safe	22.5	26	27.4

Chicken

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	6.2	62.5	26.7	2	0.9

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	12.4	10.4	14.1
1-3 times per week	37.7	41.9	37.4
4-6 times per week	31.9	30.9	28.8
7+ times per week	14.4	13.6	15.3
Never Safe	2	1.7	2

Canned food

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	14.4	14.7	28.7	29.6	11.8

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	39.8	40	38.9
1-3 times per week	30.4	30.1	28.2
4-6 times per week	7.3	8.1	7.9
7+ times per week	6.4	4.3	4.8
Never Safe	15	16.3	18.8

Fresh fruit

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	1.7	93	4	0.3	0.3

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	1.6	1.2	1.2
1-3 times per week	5.4	4.5	5.3
4-6 times per week	18.6	19.4	16.4
7+ times per week	74.1	74.4	76.3
Never Safe	0.2	0.2	0.2

Tuna

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	12.7	18.9	21.6	22.5	22.8

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	30.4	31.6	33.5
1-3 times per week	25	23.9	21.7
4-6 times per week	10.5	8.8	8.1
7+ times per week	5.3	4.5	5
Never Safe	27.8	29.5	29.9

Sweet desserts

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	12.1	34.4	34.9	15.7	1.7

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	29.1	33.2	34.9
1-3 times per week	42.3	42.2	40.9
4-6 times per week	15.8	13.3	11.8
7+ times per week	8.2	7.4	7.8
Never Safe	3.7	2.6	3.4

Frequency data for the perception of food safety during the entire pregnancy and within each trimester. Values expressed as percent of total respondents.

Table 9. *Frequency data for perception of beverages*

Tap Water

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	10.4	20.3	23.4	26.5	18.3

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	20.2	19.2	19.2
1-3 times per week	14.6	15.7	14.4
4-6 times per week	12.7	11.3	11.9
7+ times per week	26.7	26.8	26.5
Never Safe	24.7	25.4	26.4

Energy Drinks

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	3.6	1.2	2.9	12.6	78.3

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	15	11.6	11
1-3 times per week	5.1	4.2	3.7
4-6 times per week	1.1	1.1	0.9
7+ times per week	0.5	0.3	0.6
Never Safe	77.5	81.6	81.4

Wine

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	4.5	1.1	5.4	14.6	73.3

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	17.2	14.3	14
1-3 times per week	4.2	2.6	2.9
4-6 times per week	0.5	0.5	0.2
7+ times per week	0.2	0	0
Never Safe	76.9	81.6	81.6

Milk

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	4.7	68.5	21.9	3.9	0.5

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	6.7	5.7	5.6
1-3 times per week	20	20.3	20.8
4-6 times per week	33.6	34	32.7
7+ times per week	38.6	38.1	38.6
Never Safe	0.9	1.4	2

Coffee

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	7.4	2	6.5	35.2	47.6

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	31.6	30.4	28.4
1-3 times per week	14.6	10.5	9.1
4-6 times per week	2.6	1.9	2
7+ times per week	0.5	0.3	0.3
Never Safe	50.1	56	58.3

Beer

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	1.6	0.3	0.6	2.6	93.8

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	4.3	2.6	3.1
1-3 times per week	1.7	1.1	1.1
4-6 times per week	0.2	0.5	0.2
7+ times per week	0	0	0.2
Never Safe	92.6	94.9	94.3

Juice

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	6.2	62.8	27	2.2	0.2

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	5.6	6	6.5
1-3 times per week	28.4	28.1	27.8
4-6 times per week	32.2	32.9	32.6
7+ times per week	31.5	30.5	30.4
Never Safe	0.8	0.8	0.9

Tea

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	14.7	29.8	31.6	15	7.6

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	27.6	27.8	29.9
1-3 times per week	29.8	31.8	28.4
4-6 times per week	15.5	14.3	14
7+ times per week	14.1	12.9	12.4
Never Safe	12.1	12.6	14.1

Mixed alcoholic drinks

Percent	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
	1.1	0.3	0.5	1.4	95.8

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	1.9	1.4	1.2
1-3 times per week	1.2	0.8	1.1
4-6 times per week	0.2	0.3	0.2
7+ times per week	0.2	0.2	0.2
Never Safe	95.7	96.1	96.4

Frequency data for the perception of drink item safety during the entire pregnancy and within each trimester. Values expressed as percent of total respondents.

Table 10. *Frequency data for perception of medications*

Non-prescription cold medication

	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
Percent	8.5	1.6	9	36.1	43.6

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	37.4	32.1	32.1
1-3 times per week	5.6	3.6	3.6
4-6 times per week	1.7	1.4	1.4
7+ times per week	0.2	0	0
Never Safe	54.1	61.6	61.6

Aspirin

	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
Percent	13.2	3.3	18	30.5	34.9

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	43.6	43.9	43.9
1-3 times per week	9	7.1	7.1
4-6 times per week	2.6	1.2	1.2
7+ times per week	0.3	0.2	0.2
Never Safe	43.7	47.4	47

Prenatal vitamins

	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
Percent	10.4	63.3	19.1	3.3	2.5

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	13.8	14.1	14.6
1-3 times per week	19.7	21.6	20.8
4-6 times per week	22.5	21.7	20
7+ times per week	37.4	36.1	36.7
Never Safe	5.3	5	6.2

Frequency data for the perception of medication safety during the entire pregnancy and within each trimester. Values expressed as percent of total respondents.

Tylenol

	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
Percent	12.1	4.7	21.2	35.5	26.4

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	48.2	46.8	46.8
1-3 times per week	10.5	7.6	7.6
4-6 times per week	2	0.9	0.9
7+ times per week	0.6	0.5	0.5
Never Safe	37.8	41.6	43.3

Anti-depressants

	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
Percent	8.4	0.5	3.3	22.5	65

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	19.1	16	16
1-3 times per week	5.4	4.8	4.8
4-6 times per week	1.4	1.1	1.1
7+ times per week	1.4	1.6	1.6
Never Safe	71.8	74.7	75.5

Advil

	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
Percent	12.2	3.9	20.5	33	29.5

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	44.7	43.9	43.9
1-3 times per week	10.9	7.8	7.8
4-6 times per week	2.5	1.6	1.6
7+ times per week	0.5	0.2	0.3
Never Safe	40.3	44.3	45.1

Prescription pain medication

	No Opinion	Safe	Somewhat Safe	Somewhat Unsafe	Unsafe
Percent	9.9	11	19.7	23.7	35

	1st Trimester	2nd Trimester	3rd Trimester
1-3 times per month	35.5	34.1	34.1
1-3 times per week	17.4	14.9	14.9
4-6 times per week	4.8	4.3	4.3
7+ times per week	1.6	1.4	1.6
Never Safe	40	41.6	44.5

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