

The Effects of Prenatal Alcohol and Nicotine Exposure on Later-Life Drug-Seeking Behavior and Memory Performance

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The Effects of Prenatal Alcohol and Nicotine Exposure on Later-Life Drug-Seeking Behavior
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Abstract

Alcohol and nicotine are the two most commonly consumed licit substances among pregnant individuals, and the potential consequences of prenatal exposure to each drug include a variety of neurological and behavioral alterations. These may include an increased likelihood of developing a substance use disorder (SUD) and/or cognitive impairments later in life. However, 30% of pregnant individuals who report consuming alcohol also report consuming nicotine, yet the potential consequences of combined prenatal exposure are not understood. Furthermore, 40% of pregnancies in the U.S. are unplanned, meaning that drug exposure most commonly occurs during the first trimester. However, there is limited research investigating whether combined prenatal exposure to alcohol and nicotine may exacerbate behavioral alterations more than individual prenatal exposures, particularly with early pregnancy exposure alone. This study examined whether co-exposure to alcohol and nicotine via electronic cigarettes during the first trimester increases drug-seeking behaviors and/or impairs memory performance more than individual exposures using a rat model. Pregnant Sprague-Dawley rats were exposed to alcohol, nicotine, the combination, or an e-cigarette vehicle from gestational days (GD) 5-11, mimicking the human first trimester. Following birth, adolescent offspring were examined for nicotine-seeking behaviors using a self-administration vapor paradigm. Offspring were also examined for memory performance using a novelty object recognition paradigm during both adolescence and adulthood. Results indicated that neither prenatal alcohol and/or nicotine via e-cigarettes altered drug-seeking behaviors, although females exhibited more drug-seeking behaviors compared to males. Additionally, combined prenatal exposure did not alter memory performance in either adolescence or adulthood.

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Introduction

Prenatal Alcohol Exposure

Alcohol is one of the most common licit substance consumed by pregnant individuals; in fact, 13.5% of pregnant individuals reporting alcohol consumption with 5.2% report binge drinking (Walter et al., 2023). Alcohol exposure in utero is associated with a variety of abnormalities including birth defects, (Harrison et al., 2008) physical and cognitive impairments (Scott-Goodwin et al., 2016), and neurodevelopmental disabilities (Walter et al., 2022) known as fetal alcohol spectrum disorders (FASD). FASD affects 5% of individuals in America (Scott-Goodwin et al., 2016, Walter et al., 2023, Flannigan et al., 2020) with potential lifelong brain and body adverse consequences resulting from high levels of prenatal alcohol exposure (Flannigan et al., 2020). In certain cases, individuals require ongoing support with their daily living experiences and without adequate support, can experience adverse outcomes throughout their life.

In addition to physical and cognitive impairments, prenatal alcohol exposure can lead to neurological alterations. Research has shown that any drug exposure in utero may lead to abnormalities in the reward processing system, specifically the forebrain regions, within preclinical models (Malanga et al., 2003). Alcohol specifically increases synaptic dopamine in the nucleus accumbens by increasing neuronal firing within the ventral tegmental area. These exacerbations within the reward processing system increase the likelihood of drug abuse, independent of genetics and parental and environmental influences seen within clinical models (Glantz et al., 2006). This may be due to the neuronal network becoming sensitized to the intoxicating effects in utero, resulting in an increased preference for alcohol and other substances (Dodge et al., 2022).

Prenatal alcohol exposure can additionally lead to cognitive impairments. These impairments include effects on learning, visual spatial capacities, attention, concentration, and working memory (Danel, 2014). More specifically, alcohol exposure in utero can lead to severe impairments in executive functioning including dysexecutive syndrome in clinical models. These impairments are often overlooked because of misdiagnosis and treated through psychiatry as attention and/or mood disorders, yet prenatal exposure to alcohol is rarely taken into consideration as the cause (Danel, 2014). Additionally, prenatal alcohol exposure can result in central nervous system impairments, neurodevelopmental abnormalities including verbal and visual-spatial learning, and reaction time (Glantz and Chambers, 2006).

Central nervous system (CNS) abnormalities associated with prenatal alcohol exposure include decreased brain volume and impairments in learning, attention, reaction time, and executive functioning (Glantz et al., 2006). Preclinical models using magnetic resonance imaging (MRI) showed reduced hippocampal volume in the right hemisphere and shape changes in mice prenatally exposed to alcohol (Solar et al., 2022). Additionally, alterations within oligodendrocyte generation and myelination within the hippocampus have been observed within Solar's model, and poor performance on hippocampus related tasks. This suggests long-term learning and memory impairments resulting from prenatal alcohol exposure.

Similar observations have been observed in clinical models, with hippocampal reduction in the neonate period that continued into adulthood. Individuals exposed to alcohol prenatally have 5%-25% smaller hippocampal volume compared to non-exposed individuals, as seen in MRI studies (Solar et al., 2022). Additionally, shape abnormalities have been observed within children; however, these shape changes have only been observed within the head and the tail of the hippocampus suggesting these abnormalities may be region specific (Solar et al., 2022).

There is also evidence of hippocampal volume reductions being dose-dependent, showing more reductions within children with fetal alcohol spectrum disorder compared to children that have been prenatally exposed to alcohol but do not have this diagnosis (Biffen et al., 2020).

Prenatal Nicotine Exposure

Similar to alcohol, nicotine is also a commonly consumed licit substance used by pregnant individuals. Depending on the survey measure, an estimated 5-26% of pregnant individuals use nicotine throughout their pregnancy (Scott-Goodwin et al., 2016). Importantly, prenatal nicotine exposure may lead to physical alterations among offspring as early as the seventh week of pregnancy (Scott-Goodwin et al., 2016). Electronic cigarette (e-cigarette) use has become increasingly popular throughout the years as a presumed safe alternative to traditional cigarettes, despite the lack of evidence to support the safety mechanisms (Kim et al., 2020).

More than 10% of pregnant individuals have used e-cigarettes, despite scientific literature suggesting that any amount of nicotine consumption during pregnancy is dangerous to the development of the fetus. Compared to traditional cigarettes, e-cigarettes do not reduce negative neonatal outcomes and are not safer. Kim et al. (2020) conducted a study comparing the outcomes of traditional cigarette use and e-cigarette use during pregnancy and found that prenatal e-cigarette use is significantly associated with the infant being small for gestational age, having low birthweight, and being born preterm.

Similar to FASD, prenatal nicotine exposure can lead to similar outcomes with long-lasting changes in brain structure (Bassey et al., 2018). Clinical models reported cigarette smoking while pregnant can cause a wide variety of impairments in the fetus, including low fetal

growth in utero, low birth weight, sudden infant death syndrome, multiple medical disorders (Glantz et al., 2006), respiratory illness, hearing loss, and an increased risk of obesity (Scott-Goodwin et al., 2016). There is evidence suggesting dose-dependent interactions with neonate kidney volume, with higher consumption resulting in smaller kidney volume compared to low consumption (Römer et al., 2020). Smaller kidney volume results in fewer nephrons, causing hypertension and kidney disease that may develop later in life.

In addition to medical disorders, prenatal nicotine exposure is associated with cognitive and behavioral impairments (Levin et al., 1996). In preclinical models utilizing a T-maze and a radial-arm maze, adverse cognitive and behavioral effects have been observed in rats that were prenatally exposed to nicotine, suggesting impairments in learning and memory behavior. Similar effects have been observed within clinical models in children showing impairments in cognitive performance, specifically in verbal and memory performance. These individuals may be more likely to develop deficits including attention deficit hyperactivity disorder (ADHD), impaired attention and orientation, poor impulse control and learning impairments. These consequences have been shown to be long-term in the majority of studies investigating prenatal nicotine exposure (Levin et al., 1996).

Nicotine exposure in utero is also associated with an increase in offspring cigarette smoking, an increase in nicotine dependence, and an increased chance of developing substance use disorder. Prenatal nicotine exposure, which may increase the number of nicotine binding receptors within the brain, may lead to sensitization. This may increase one's preference towards nicotine (Dodge et al., 2022) and lead to altering the reward system within the brain, ultimately increasing the likelihood for offspring self-administration of nicotine.

Acute consumption of nicotine can produce positive effects on cognition involving attention, vigilance, working and associative memory in both pre-clinical and clinical models (Juárez-Portilla et al., 2018). However, the literature shows that lower doses of nicotine can enhance cognition, while higher doses of nicotine can impair cognitive functioning. Working memory relies heavily on dopamine and previous studies reported nicotinic agonist increasing the presence of dopamine causing a relationship between nicotine consumption and memory performance.

Nicotine exposure in general increases nicotinic acetylcholine receptors throughout the brain, which modulates the development of axonal pathfinding, synapse formation, and cell survival during fetal brain development (Scott-Goodwin et al., 2016). Chronic exposure causes desensitization in the receptors, causing long-term changes in receptor function. In clinical models, adolescents exposed to nicotine in utero exhibit smaller amygdala and pallidum volumes causing exacerbations in behavior and executive functioning. In addition, alterations in nicotinic receptors can cause growth restrictions, reduced newborn excitability, and unstable breathing. Instability in breathing results from these receptor alterations within the brain stem nuclei, correlating with sudden infant death syndrome (Scott-Goodwin et al., 2016).

Prenatal Co-Exposure

Alcohol and nicotine are the two most commonly used licit substances used by pregnant individuals, with 30% of pregnant individuals who report consuming alcohol also report using nicotine products (England et al., 2020). Despite this, there has been sparse research investigating the effects of co-exposure on offspring. So far, prenatal co-exposure is associated with negative impairments, such as pathological outcomes, behavioral problems, cognitive

impairments, morphological development (Römer et al., 2020), neurodevelopmental, and impairments in the development of the central nervous system (Glantz et al., 2006). Co-use during pregnancy is most prominent within the first trimester, indicating some individuals may have quit once they discovered they are pregnant (England et al., 2020).

When combined, alcohol and nicotine produce an interactive effect of reduced sensitivity to the pharmacological effects of alcohol, also known as a cross-tolerance (Doyon et al., 2013). The combination interacts with the mesocorticolimbic dopamine system, which plays the main role in reinforcement, with rodent studies showing high reinforcement in self-administration studies. Both drugs act upon the mesocorticolimbic dopamine system by increasing the firing rate of dopamine neurons in the nucleus accumbens, with co-consumption increasing firing more rapidly compared to either drug alone (Doyon et al., 2013).

Additionally, co-exposure can increase responsiveness to drugs, specifically any drug(s) that an individual was exposed to in utero. Drug exposure in utero is associated with neurodevelopmental changes within the brain, where offspring receptors may become sensitized to the drug(s) they were exposed to Dodge et al. (2022). It is possible that increased responsiveness to preference for a drug may increase the likelihood that an individual with prenatal alcohol and/or nicotine exposure may have increased reward-seeking behaviors and develop a substance use disorder (SUD) later in life.

While acute, low doses of nicotine may improve cognition, the same cannot be stated for alcohol exposure. Heavy alcohol use within clinical populations is associated with “blackouts”, where information fails to be transferred from short-term memory to long-term memory, with repeated use causing memory deficits (Juárez-Portilla et al., 2018). Furthermore, Moore et al. (2021) illustrated that prenatal alcohol exposure is heavily associated with working and spatial

working memory deficits. For example, children exposed to alcohol in utero score lower than non-exposed children on executing functioning tasks later in life. Their findings suggest that prenatal alcohol exposure may alter the progression of cognitive development when comparing working and spatial memory performance within children and adolescents; however, it is unknown if this effect is still prominent during adulthood or if the exposed individuals had a slower progression and eventually catch up in development (Moore et al., 2021).

Sex Differences

In general, sex differences have been observed in drug-seeking behaviors, with females being more likely to self-administer nicotine more rapidly than males and work harder to receive the drug (Becker et al., 2008). Women reported having shorter intervals between cigarettes and find it more difficult to quit smoking compared to men. Research investigating these differences within clinical and pre-clinical studies state that a female's menstrual cycle may exacerbate the likelihood of substance consumption, potentially causing these differences. Specifically, women experience more cravings during the late luteal phase which may increase desire to seek-out nicotine, while experiencing less anxiety during the follicular phase which may alleviate that desire (Becker et al., 2008)

On the contrary, clinical models of individuals exposed to alcohol in utero have observed more substance abuse behaviors within males compared to females (Yates et al., 1998). Males reported more symptoms that met substance use disorder (SUD) criteria compared to females, including nicotine, suggesting males prenatally exposed to alcohol are more likely to seek out nicotine compared to females. In addition, prenatal nicotine exposure is associated with adolescent nicotine use more in females compared to males, where it is suspected that prenatal

nicotine exposure alters female hormones and cravings (Rydell et al., 2012). This supports Becker's discussion with general sex differences, as hormonal changes typically occur during adolescence when a female's menstrual cycle begins. Taken together, this suggests that females prenatally exposed to nicotine are more likely to seek out nicotine compared to males.

With general sex differences in memory performance, females excel in memory performance compared to their male counterparts within preclinical models (Ghi et al., 1999). Females presented better verbal memory, performed better in an object location memory test, and showed longer lasting memory retrieval. These differences may be connected to estrogen, which boosts the expression of serotonin and increases cortex density which is associated with cognitive function (Ghi et al., 1999).

Similar sex differences have been observed after prenatal nicotine exposure, with males experiencing nicotine-induced memory deficits, and females showing memory improvements (Levin et al., 1996). Males showed a more prominent decrease in dopamine and norepinephrine from prenatal nicotine exposure compared to females, with an additional drop in male testosterone levels, ultimately diminishing memory performance within males. However, memory impairments may vary based on when prenatal exposure took place (Sikic et al., 2022). In a study comparing prenatal nicotine exposure during different developmental windows, males showed impairments when exposed during gestational day (GD)0-postnatal day (PD)7 and GD14-PD7 while females only showed impairments when exposed during GD0-PD0 and GD14-PD0. This suggests sex differences in memory performance may vary based on the trimester of exposure, which contradicts with those found for alcohol. Females exposed to alcohol in utero showed smaller hippocampal volumes in clinical models, which is associated with poorer

memory performance (Solar et al., 2022). This suggests females prenatally exposed to alcohol may exhibit impairments in memory performance compared to males.

Use of Animal Models

This research cannot be ethically conducted through the clinical population; therefore, pre-clinical rodent models are valuable. Evaluating the effects of any drug-exposure in clinical models is challenging due to confounding factors such as genetics and environmental influences, reliance on self-report data, the length of longitudinal studies, and the inability to measure drug blood levels. Preclinical models allow careful manipulation of variables that cannot be controlled within the human population such as drug dosing, environmental and/or genetic influences, and reliable data collection in real time in a reasonable timeframe. Sprague-Dawley rats are well suited to this type of experiment due to their fast development, translational findings to the clinical population, the ability to control confounding variables, and much is known about their brain and behavioral development allowing a reasonable timeframe. Therefore, a preclinical rodent model can provide quick, reliable, controllable, and translational alternative to clinical models in teratology research.

Study Design

Using a rat model, this study examined the effects of prenatal co-exposure of alcohol and nicotine via e-cigarettes on offspring drug-seeking behavior and memory performance. Pregnant rats were exposed to alcohol, nicotine, the combination, or the control condition during the human first trimester equivalent (gestational day [GD] 5-11) to mimic current consumption

patterns. Following birth, offspring were examined for nicotine self-administration during adolescence, as well as for memory performance during both adolescence and adulthood.

Materials and Methods

All procedures were approved by the WCU Institutional Animal Care and Use Committee (WCU IACUC; protocol #202401) and comply with the National Institutes of Health (NIH) Office of Laboratory Animal Welfare (OLAW) Assurance approved to WCU. All breeding and behavioral procedures took place in the Breit Lab (Dr. Kristen Breit, Department of Psychology, Wayne Hall Animal Facility, West Chester University of Pennsylvania).

Breeding

Adult Sprague-Dawley rat breeding pairs were housed overnight for up to 5 consecutive days. The presence of a seminal plug indicated mating and was designated gestational day (GD) 0.

Prenatal Exposure Group Assignment

This study used a 2 (ethanol [EtOH], Air) x 2 (nicotine [NIC], vehicle [VEH]) x 2 (female, male) design, resulting in 4 prenatal drug exposure groups represented among female and male offspring. Following the presence of a seminal plug (GD 0), pregnant rats were individually housed in a temperature and humidity-controlled environment. On this same day, pregnant rats were randomly assigned to 1 of 4 prenatal exposure groups: EtOH + NIC (combined exposure), EtOH + VEH (alcohol alone), Air + NIC (nicotine alone), or Air + VEH (vehicle control).

Prenatal Drug Exposure and Blood Measurements

Pregnant dams were exposed to their assigned drug(s) once daily from GD 5-11 for a 3-hour time period. GD 5-11 mimics the human first trimester and is the most common time period of prenatal exposure to alcohol and/or nicotine based on self-report data (England et al., 2020). Dam food intake, water intake, and body weights were monitored daily throughout the pregnancy. Dams were given 200-200.5 grams of food each day and 400 mL of water each day in order to measure how much was consumed over 24 hours. In addition, core body temperatures were recorded before and after the vapor exposure using a rectal thermometer.

A vapor inhalation chamber designed by La Jolla Alcohol Research Inc. (LJARI; La Jolla, CA) was used for prenatal drug exposure. This apparatus houses rodents in chambers similar to their home cage to eliminate stress, has a programmable control box for drug exposure customization, uses commercially available e-cigarette tanks (Geekvape Zeus Sub-Ohm Tank 5ML) and coils (Geek Vape Z Series Coil Z0.2) to maximize clinical relevance, and has an air flow meter to maximize safety.

Using the 2 (EtOH, Air) x 2 (NIC, VEH) design for prenatal exposure, half of the dams were exposed to EtOH. EtOH (95%) was administered in a constant airflow (10 L/min) at a moderate dose (3.7% EtOH air concentration) for 3 consecutive hours; target blood alcohol concentration (BAC) levels were 200mg/dL. A moderate dose was chosen to maximize clinical relevance. Subjects that did not receive EtOH instead received only airflow (Air) in the same manner and time period.

Half of the subjects exposed to EtOH or Air were exposed to Nicotine (NIC) during the last 30 minutes of the 3-hour exposure period. NIC was administered in 7 6-second puffs over 30 minutes at an airflow rate of 2L/min at 18 mg/mL, with a target plasma nicotine level of 20ng/mL. An 18 mg/mL dose of nicotine is considered moderate and is the most common dose

of nicotine e-liquid consumed today (Manigrasso et al., 2015). Subjects that did not receive nicotine instead only received the e-cigarette vehicle (30% propylene glycol, 70% vegetable glycerin) in the same manner and time-period. An additional 10 minutes of airflow was administered for drug clearance at the end of the 3-hour exposure period.

Blood samples (200 uL) were collected from all dams via tail snip on the first (GD5) and last day (GD11) of prenatal drug exposure to confirm target drug levels in the blood and examine potential pharmacokinetic effects of the co-exposure of alcohol and nicotine. Tail snips involved cutting the tip of the tail with a razor blade and collecting the blood using a capillary tube, with antibacterial ointment being applied after collection. Blood samples were spun to extract plasma for 10 minutes utilizing a centrifuge, with 150uL of plasma for blood alcohol analyses and 50uL of plasma for nicotine analyses. BAC levels were analyzed in the Breit Lab using an Analox Alcohol Analyzer (4Model AMI; Analox Instruments; Lunenburg, MA). Nicotine metabolite levels will be analyzed by Dr. Nina Ossanna at MZ Biolabs (Tucson, AZ) at a future time.

Following the prenatal vapor exposure paradigm, dams were monitored daily until birth (usually GD 22).

Offspring Behavioral Group Assignment

The day of birth was designated as postnatal day (PD) 0. On PD 1, litters were culled to 8 offspring (4 females and 4 males whenever possible). To avoid confounds and litter effects in behavioral data collection, only one sex pair per litter was used for each behavioral measure within the two behavioral paradigms, creating Groups 1-4. Groups 1 and 2 were examined for drug-seeking behaviors during adolescence; Group 1 was trained to self-administer nicotine (NIC) via an e-cigarette (18 mg/mL), while Group 2 was trained to self-administer the e-cigarette

vehicle (VEH; 30% propylene glycol, 70% vegetable glycerin) and serve as a control group for any potential confounds regarding self-administering vapor in itself. Groups 3 and 4 were examined for memory performance; Group 3 was examined for memory performance during adolescence, while Group 4 was examined for memory performance during adulthood.

On PD1, all offspring were tattooed on their paws for identification purposes. All pups were monitored daily for body weight, body lengths, and developmental milestones. Developmental milestones include ear unfurling, eye opening, and teeth eruption till PD20. All subjects were weaned from the dam on PD21, where food was placed on the bottom of the cage to ensure food consumption in case they had not learned to reach their food from the topper. On PD28, all pups were separated by sex; beginning on this day, any pups in Groups 1 or 2 had their food, water, and body weight measurements taken daily to collect baseline data prior to the self-administration paradigms. Offspring were given 200-22.5 grams of food each day and 400 mL of water each day in order to measure how much was consumed over 24 hours.

Offspring Groups 1 and 2: Self-Administration of Nicotine or Vehicle

The self-administration paradigm took place once per day for 1 hour over 15 consecutive days beginning on PD 30 (PD 30-44), which mimics human early adolescence. The same vapor inhalation apparatus (LJARI) was used for the self-administration paradigm, utilizing the self-administration mode of the machine. Two nose-poke holes are located in each chamber, which contain sensors that administer NIC (Group 1, 18 mg/mL) or the VEH (Group 2).

Throughout the paradigm, subjects in Groups 1 and 2 underwent a fixed-ratio (FR1) schedule where subjects receive one puff of vapor each nose poke. When the nose poke hole was poked successfully (valid nose poke), a 6-second puff of NIC (Group 1) or VEH (Group 2) was

administered, followed by a light prompt with a 10-second timeout period. The light prompt acted as a cue for subjects to associate the light with the vapor. The apparatus tablet recorded the number of valid nose-pokes, total nose-pokes, and total number of vape deliveries.

Blood samples (100mL) were collected via tail snip on PD35 and PD44 for nicotine analyses to confirm how much nicotine was present in subjects' blood on the first and last days of the paradigm. Nicotine metabolite levels will be analyzed by Dr. Nina Ossanna at MZ Biolabs (Tucson, AZ) at a future date.

Group 3-4: Novelty Object Recognition Early Adolescence and Adulthood

The novel object recognition (NOR) task was utilized to measure rodent's memory performance beginning on PD 30-31 (Group 3, adolescence) or PD 60-61 (Group 4, adulthood). The NOR paradigm utilizes a rodent's natural increase in interaction to novel objects while interacting less with objects that it remembers. The NOR apparatus consists of a clear Plexiglas box (20in x 20in) and three novelty objects: two yellow Lego blocks and a blue and black round soccer ball fidget spinner. To acclimate, subjects were placed in an isolated holding cage (26cm x 47.5cm) in the testing room for 30 minutes to mitigate potential stress. The room was illuminated by a lamp to diminish potential stress, as they are nocturnal by nature. A video camera was placed above the apparatus to record the subject's interactive behavior and interaction time to each object, which was later manually coded by 2 separate naive coders using ODLog.

On the first day of testing (PD 30 [Group 3] or PD 60 [Group 4]), the 2 identical objects (yellow Lego blocks) were placed on opposite ends of the box. Subjects were then placed in the box and allowed to roam freely for 10 minutes with a camera above recording their interaction.

On the second day of testing (PD 31 or 61), 1 of the yellow Lego blocks was placed on the left side of the cage, and the second yellow Lego block was replaced with the 3rd object (blue and black round soccer ball fidget spinner). Subjects were then placed in the box and allowed to roam freely for 10 minutes with a camera above recording their interaction. Interaction behaviors will be coded by 2 naive coders using ODLog, including sniffing, climbing, and biting.

An inter-rater reliability score of 90% or higher was required from the 2 naive coders for all NOR data, and data were averaged between the 2 coders.

Tissue Collection and Harvesting

Offspring and dam brain, heart, liver, and lung tissue were collected within 7 days of weaning (PD 21) for dams and within 7 days behavioral test completion for offspring. All subjects were individually euthanized via medical grade carbon dioxide (CO₂) for 5 minutes, exposed to residual carbon dioxide for 5 minutes, and then decapitated via guillotine to ensure proper two-point euthanization. All organs were weighed and frozen for future analyses.

Expected Results

Although literature examining the effects of prenatal co-exposure to alcohol and nicotine via e-cigarettes is sparse, we hypothesized our expected results based on the literature examining individual prenatal exposures to alcohol or nicotine (separately). Based on previous research and the collection of pilot data within our laboratory (Breit et al., 2022), we did not predict any mortality or pregnancy issues with maternal dams during drug exposure.

Previous research has shown that separately, prenatal alcohol exposure or prenatal nicotine exposure increase drug-seeking behaviors (Glantz et al., 2006; Malanga et al., 2003;

Dodge et al., 2022). Both prenatal alcohol and nicotine exposure, separately, alter the reward processing system and increase dopamine activity (Doyon et al., 2013; Malanga et al., 2003; Dodge et al., 2022). Co-exposure to alcohol and nicotine may exacerbate the reward processing system, which in turn may increase an individual's drug-seeking behavior, independent of genetic or environmental influences (Glantz et al., 2006). This suggests that independently, prenatal alcohol and nicotine exposure might increase nicotine-seeking behavior within offspring.

Given that alcohol and nicotine have interactive mechanistic properties (Doyon et al., 2013), it was predicted that the combined prenatal exposure would increase nicotine-seeking behaviors more than singular prenatal exposure to either drug. Both nicotine and alcohol target the same brain areas, and given that the combination may increase dopamine activity more than either drug alone, the combination may be more reinforcing (Doyon et al., 2014).

Within clinical models, opposite sex differences in drug-seeking behavior have been observed in individuals exposed to alcohol or nicotine in utero. Males who have been prenatally exposed to alcohol have reported more symptoms that met substance use disorder (SUD) criteria compared to females who have been prenatally exposed to alcohol (Yates et al., 1998). In contrast, adolescent women exposed to nicotine in utero consume nicotine more frequently compared to men who have also been prenatally exposed to nicotine (Rydell et al., 2012). There is a lack of notable sex differences within individuals who have been prenatally exposed to both alcohol and nicotine, and with the contradictions of singular exposure effects, it was predicted that sex differences might not be observed following prenatal co-exposure to alcohol and nicotine.

Separately, prenatal alcohol and nicotine exposure are associated with cognitive impairments (Levin et al., 1996; Danel et al., 2014; Solar et al., 2022; Biffen et al., 2020). MRI studies showed reduced hippocampal volume with individuals exposed to alcohol in utero, which is associated with learning and memory impairments, and observed poor performance in learning and attention (Glantz et al., 2006; Solar et al., 2022). Prenatal nicotine exposure is associated with similar impairments, with individuals exposed in utero exhibiting poor cognitive performance, specifically in memory (Levin et al., 1996). This suggests that independently, prenatal alcohol and nicotine exposure might increase memory impairments. It was predicted that the combined prenatal exposure would increase memory impairments more than singular exposure to either drug. In addition, adolescence is considered a sensitive period in brain development, with grey matter decreasing and white matter increasing due to synaptic pruning (Juárez-Portilla et al., 2018). Due to constant synaptic pruning on the developing brain during adolescence, it was predicted that adolescence would exhibit poorer memory performance compared to adults regardless of prenatal exposure.

Clinical models investigating effects of individual prenatal exposure to alcohol or nicotine on the timing of puberty indicated no significant associations with prenatal alcohol exposure on early/delayed onset of puberty (Brix et al., 2019). However, studies investigating prenatal nicotine exposure effects on the onset of puberty indicated that individuals with prenatal nicotine exposure experienced early puberty in both males and females (Brix et al., 2018). Puberty typically occurs on PD 30 for females and PD 40 for males, suggesting that puberty may potentially begin during memory testing. Taking potential early development into account, it may be possible that adolescent subjects with prenatal nicotine exposure might exhibit less

impaired memory performance compared to subjects prenatally exposed to alcohol alone with the possibility of an earlier onset of puberty, particularly within females.

Sex differences in prenatal alcohol or nicotine exposure on memory performance have been inconsistent throughout the literature (Levin et al., 1996; Sikic et al., 2022). However, smaller hippocampal volumes have been observed within females exposed to alcohol in utero which is associated with poor memory performance (Solar et al., 2022), suggesting that memory impairments may be more prominent within females with prenatal alcohol exposure. On the contrary, males exposed to nicotine in utero exhibited poorer memory performance later in life compared to females exposed to nicotine in utero (Levin et al., 1996); however, sex differences may vary on the developmental timeline of exposure (Sikic et al., 2022). In Sikic's article investigating prenatal nicotine exposure on memory performance, females and males showed no difference in impairments during GD 0-GD 13. Since prenatal drug exposure takes place GD 5-GD 11 in the current model, we predicted that sex differences might not be observed following prenatal nicotine exposure alone. It was predicted that only singular prenatal alcohol exposure and the combined exposure would exhibit sex differences within memory performance.

Results

Dam Measurements

A total of 34 dams were exposed to either alcohol alone (EtOH+VEH, $n = 8$), nicotine alone (Air+NIC, $n = 9$), the combination (EtOH+NIC, $n = 12$), or the control vehicle (Air+VEH, $n = 5$). However, 14 dams total were excluded from analyses due to unsuccessful pregnancies (EtOH+VEH: $n = 5$, Air+NIC: $n = 3$, EtOH+NIC: $n = 5$, Air+VEH: $n = 1$), leaving a total of 20 pregnant dams for analyses (EtOH+VEH: 3, Air+NIC: 6, EtOH+NIC: 7, Air+VEH: 4). Dam body weight, food, and water measurements took place daily throughout pregnancy, from gestational days (GD) 0-22. Drug exposure took place daily during GD 5-11, with their internal temperatures recorded before and after drug exposure each day.

Body Weight

All dam measurements (body weight, food intake, water intake) were analyzed using a 2 (EtOH, Air) $\times$ 2 (NIC, VEH) repeated measures ANOVA during GD 0-22. Body weight analyses indicated no interactions or main effects of EtOH or NIC exposure overall; however, a 2-way interaction of Day*NIC was observed ($F[22,440] = 3.85$, $p < 0.001$).

Follow-up analyses examining dam body weight and exposure groups on individual days exhibited no main effects of nicotine; however, there was a main effect of EtOH exposure during GD 17-21 ($ps < 0.05$) only (*figure 1*). A 2 (EtOH, Air) $\times$ 2 (NIC, VEH) repeated measures ANOVA for GD 17-21 specifically to confirm this main effect yielded a main effect of EtOH ($F[1,20] = 6.47$, $p = 0.019$), where dams exposed to any EtOH (EtOH+NIC, EtOH+VEH) weighed less than those exposed to any Air (Air+NIC, Air+VEH).

Taken together, these data suggests that the Day*NIC interaction was due to variability in body weight gain between exposure group types without meaningful differences. These data also suggest that any type of alcohol exposure (EtOH+NIC, EtOH+VEH) during early pregnancy may reduce body weight gain compared to dams with no alcohol exposure (Air+NIC, Air+VEH), even after alcohol exposure has ceased.

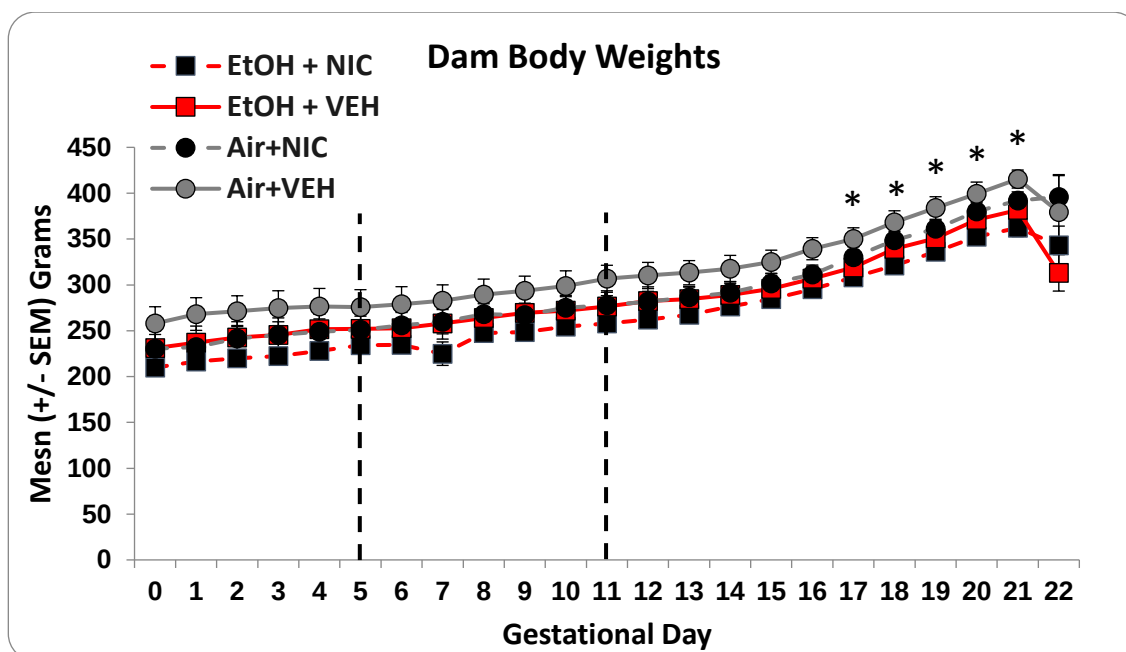


Figure 1: Dams with any alcohol exposure weighed less than dams with no alcohol exposure during GD 17-21. *EtOH < Air, $F[1,20] = 6.47$, $p = 0.019$.

Dam Food Intake

Dam food intake analyses indicated no main effects of EtOH or NIC exposure; however, the 2 (EtOH, Air) x 2 (NIC, VEH) repeated measures ANOVA for all days (GD 0-22) indicated a 2-way interaction of Day*NIC ($F[22,440] = 2.77$, $p < 0.001$).

Follow-up analyses examined individual days of dam food intake to further investigate this interaction. These analyses indicated that any NIC-exposed dams (EtOH+NIC, Air+NIC) ate

more than their VEH counterparts (EtOH+VEH, Air+VEH) on GD1 (pre-vape exposure), GD5, GD14, GD15, GD16, and GD21 ($p < 0.05$, *figure 2*), suggesting that pregnant dams exposed to nicotine alone or the combination generally ate more compared to pregnant dams not exposed to nicotine. This effect was particularly observed after exposure had ceased (later in pregnancy) which suggests post- nicotine use may increase appetite amongst pregnant dams. In contrast, EtOH exposure did not impact food intake.

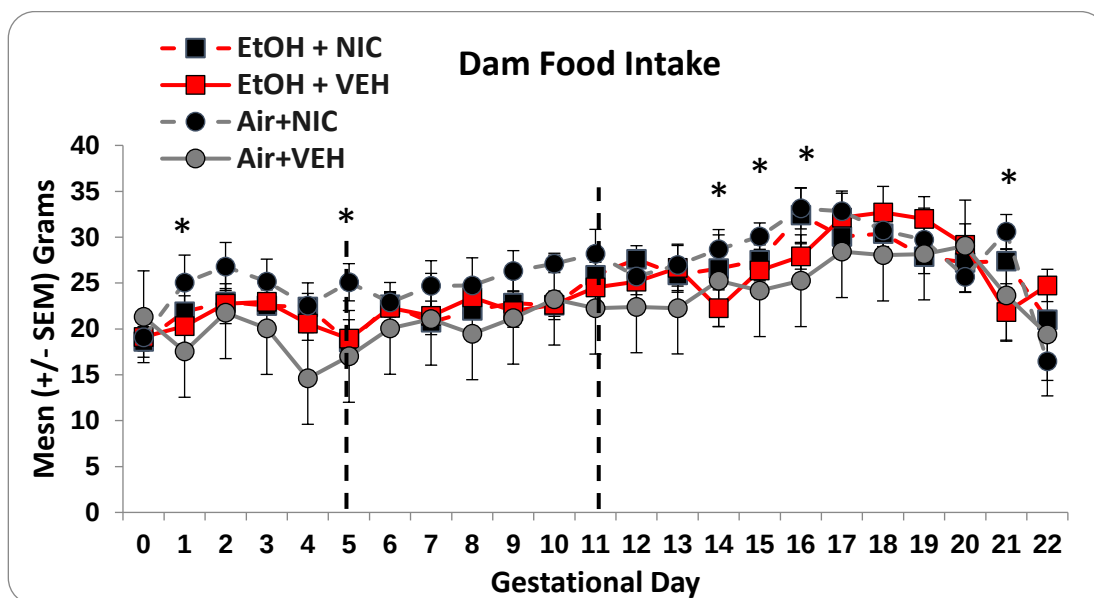


Figure 2: Dams with any nicotine exposure (EtOH+NIC, Air+NIC) ate significantly more compared to dams with no nicotine exposure (EtOH+VEH, Air+VEH). *NIC > VEH, $F[22,440] = 2.77, p < 0.001$.

Dam Water Intake

Dam water intake was analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) repeated measures ANOVA for all days (GD 0-22). Analyses indicated no main effects or interactions of EtOH or NIC across days. This suggests that neither EtOH nor NIC exposure alters water intake during pregnancy.

Dam Temperature

Pregnant dams had their temperature taken pre- and post-vaporized drug exposure during GD 5-11 to analyze physiological effects of drug exposure on core body temperature. All data (pre-temperature, post-temperature, and temperature change) was analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) repeated measures ANOVA for GD 5-11 only.

The pre-vaporized drug exposure core body temperatures for pregnant dams on GD5 were analyzed separately, since this measurement was taken to examine baseline temperatures, since dams were still naive to both EtOH and NIC during this measurement. There were no differences among dams in each exposure group on GD 5, before the prenatal drug exposure paradigm began.

Analyses for dam pre-vaporized drug exposure temperatures from GD 6-11 indicated an overall main effect of nicotine exposure on pregnant dams ($F[1,20] = 9.75, p = 0.005$) (*figure 3*), where pregnant dams with any nicotine exposure (EtOH+NIC, Air+NIC) had generally lower core body temperatures compared to those with no nicotine exposure (EtOH+VEH, Air+VEH). Dam temperature changes were analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) repeated measures ANOVA for GD 5-11. Analyses indicated a main effect of nicotine exposure, where dams exposed to any nicotine (EtOH+NIC, Air+NIC) experienced significantly less core body temperature changes ($F[1,20] = 12.64, p = 0.002$) (*figure 4*) during the vape exposure with no differences amongst days. This suggests that pregnant dams with any type of nicotine exposure (EtOH+NIC, Air+NIC) had significantly less drastic temperature changes compared to pregnant dams with no nicotine exposure (EtOH+VEH, Air+VEH), which is likely due to the fact that

their pre-vaporized drug exposure body temperatures were already lower before each session began.

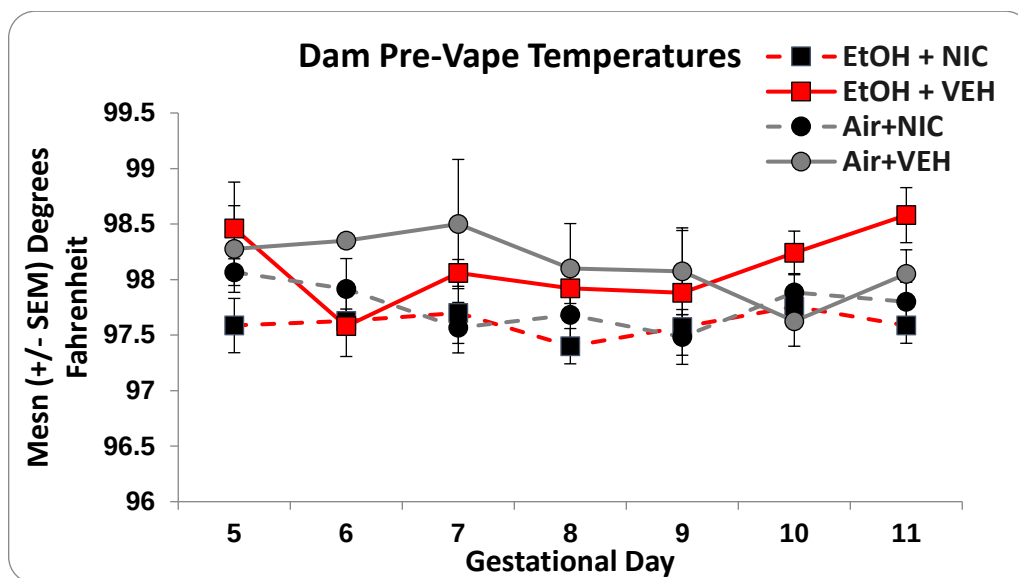


Figure 3: Dams exposed to any nicotine (EtOH+NIC, Air+NIC) had lower core body temperature compared to dams with no nicotine exposure (EtOH+VEH, Air+VEH) prior to drug exposure. $\text{NIC} < \text{VEH}$, $F[1,20] = 9.75$, $p = 0.005$.

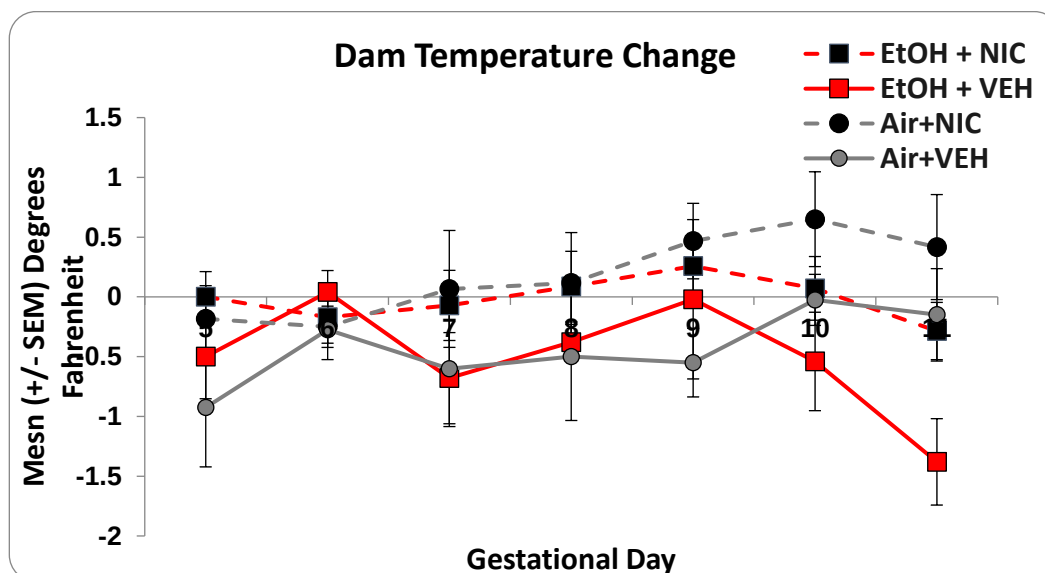


Figure 4: Dams exposed to any nicotine (EtOH+NIC, Air+NIC) had less drastic temperature change compared to dams with no nicotine exposure (EtOH+VEH, Air+VEH). $\text{NIC} < \text{VEH}$,

$$F[1,20] = 12.64, p = 0.002.$$

Dam Pup Outcomes

A 2 (EtOH,Air) x 2 (NIC, VEH) ANOVA was used to analyze dam gestational day (GD) length, the number of pups born, the sex ratio of pups, the number of females born, and number of males born. Analyses indicated no significant differences in GD length, number of pups born, sex ratio, or total number of males born among prenatal exposure groups. However, a trending interactive effect of EtOH*NIC was observed in the total number of females born ($F[1,20] = 3.66, p = 0.07$) with follow-up analyses indicating that dams exposed to nicotine alone (Air+NIC) had significantly fewer female offspring compared to pregnant dams in the control condition (Air+VEH; ($F[1,9] = 4.95, p = 0.053$)). This suggests that pregnant dams exposed to nicotine alone (Air+NIC) may have less females born compared to control conditions, but is not statistically significant.

Dam Tissue

Dam tissue was collected once pups were weaned from the mother, where dams' body, brain, liver, heart, and lung weights were recorded. Weights were analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) ANOVA, which indicated no main effects or interactions of EtOH or NIC exposure or interactions in body, liver, heart, and lung weights. However, analyses indicated a main effect of nicotine exposure on dam brain volume ($F[1,24] = 4.29, p = 0.049$), where dams with any nicotine exposure during pregnancy (EtOH+NIC, Air+NIC) had higher brain volume

compared to dams with no nicotine exposure during pregnancy (*figure 5A*). This suggests that exposure to nicotine alone (Air+NIC) or the combination (EtOH+NIC) on pregnant dams during the first trimester may result in higher total brain volume compared to pregnant dams with no nicotine exposure (EtOH+VEH, Air+VEH).

A 2 (EtOH, Air) x 2 (NIC, VEH) ANOVA was utilized to investigate dam brain to body weight ratio. Analyses indicated a main effect of nicotine ($F[1,23] = 6.27, p = 0.020$), where dams exposed to nicotine (EtOH+NIC, Air+NIC) during early pregnancy had a significantly higher brain to body weight ratio compared to dams with no nicotine exposure (EtOH+VEH, Air+VEH) during early pregnancy (*figure 5B*). This further supports that dams exposed to any nicotine (EtOH+NIC, Air+NIC) during early pregnancy may have greater brain volume (and brain to body weight ratio) compared to dams not exposed to nicotine (EtOH+VEH, Air+VEH).

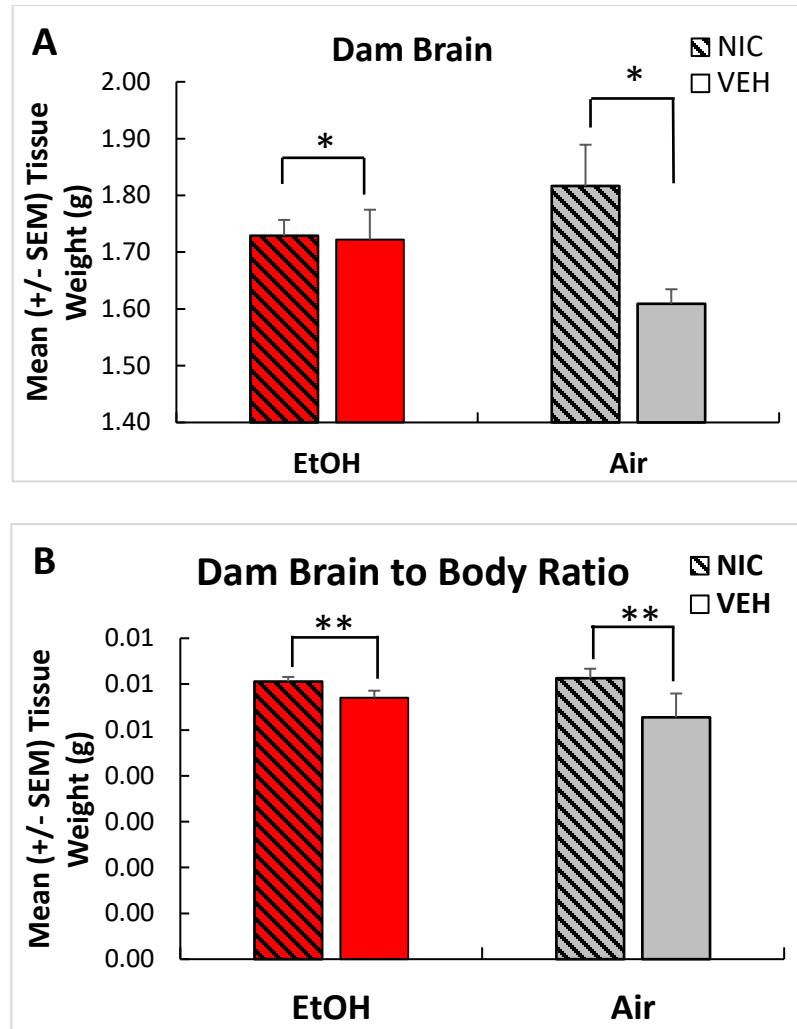


Figure 5: Dams with any nicotine exposure (EtOH+NIC, Air+VEH) had higher brain volume (A) and brain to body weight ratios (B) compared to dams with no nicotine exposure (EtOH+VEH, Air+VEH). A: *NIC > VEH, $F[1,24] = 4.29$, $p = 0.049$. B: *NIC > VEH, $F[1,23] = 6.27$, $p = 0.020$.

Pup Measurements

A total of 145 pups (EtOH+NIC: 49 [25 females, 24 males], EtOH+VEH: 23 [12 females, 11 males], Air+NIC: 41 [19 females, 22 males], Air+VEH: 32 [16 females, 16 males]) were included in analyses. All litters were culled down to 8 total pups, with 4 females and 4

males if possible, and received paw identification tattoos on PD 1. Their body weight, body length, and developmental milestones were recorded daily from postnatal day (PD) 1-20

Offspring Body Weights

Once born, offspring body weights were analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) repeated measures ANOVA across all days (PD 1-20). Since rodents are known for sex differences, individual sexes were analyzed *a priori*. Results indicated a 3-way interaction of Day*EtOH*NIC, a 2-way interaction of EtOH*NIC and a main effect of alcohol exposure ($ps < 0.05$) where pups with any prenatal alcohol exposure (EtOH+NIC, EtOH+VEH) weighed significantly less compared to pups with no prenatal alcohol exposure (Air+NIC, Air+VEH) overall.

Follow-up analyses initially split data by alcohol exposure (EtOH, Air) indicated a main effect of nicotine within EtOH-exposed offspring, where pups with combined prenatal exposure (EtOH+NIC) generally weighed significantly less compared to pups with alcohol exposure only (EtOH+VEH), regardless of sex ($F[1,68] = 36.68, p < 0.001$) (*figure 6*). Additional analyses using a 4 (EtOH+NIC, EtOH+VEH, Air+NIC, Air+VEH) x 2 (female, male) repeated measures ANOVA across GD 1-20 also yielded a Day*ExposureGroup interaction ($F[57, 2603] = 9.48, p < 0.001$) and confirmed that offspring exposed to prenatal EtOH+NIC weighed less than all other groups (SNK: $p < 0.05$). These results suggest that combined prenatal exposure to EtOH and NIC may decrease offspring developmental body weight growth more compared to individual exposure.

Importantly, effects of prenatal EtOH and/or NIC exposure were analyzed on each Day. The effects described above were observed consistently beginning on PD 3 ($ps < 0.05$). Given

that litters were culled on PD 1, it is expected that variations in litter size may shield drug-related changes in body weights initially, becoming more evident as litters become a consistent size across exposure groups ($n = 8$, 4 females, 4 males whenever possible). Taken together, combined prenatal exposure of EtOH and NIC altered body weight growth by decreasing the trajectory of body weight gain compared to individual exposure. This effect became more prominent the more pups developed.

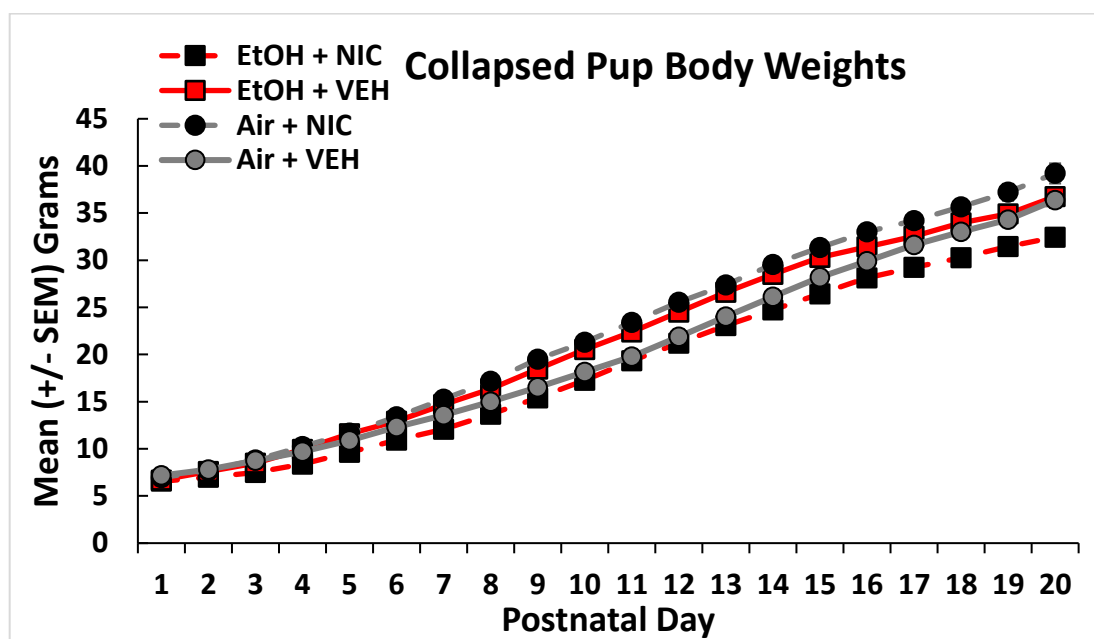


Figure 6: Offspring with combined prenatal exposure (EtOH+NIC) weighted significantly less compared to all other exposure groups. EtOH+NIC < Air+VEH < EtOH+VEH, Air+NIC, $F[57, 2603] = 9.48$ $p < 0.001$, SNK: $p < 0.05$.

Offspring Body Length

Pup body length was analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) repeated measures ANOVA across all days (PD 1-20). Results indicated a main effect of sex, a

main effect of alcohol, a main effect of nicotine, and a 2-way interaction of EtOH*NIC (p s < 0.05).

Follow-up analyses revealed that the effects of prenatal EtOH and/or NIC exposure on body length were similar to those on body weight. All data were initially split by prenatal alcohol exposure, which indicated a main effect of nicotine within EtOH-exposed offspring, where pups with combined prenatal exposure (EtOH+NIC) had significantly shorter body lengths compared to pups with alcohol exposure only (EtOH+VEH) ($F[1,68] = 42.93, p < 0.001$). A 4 (EtOH+NIC, EtOH+VEH, Air+NIC, Air+VEH) x 2 (female, male) repeated measures ANOVA analysis across PD 1-20 also yielded a Day*ExposureGroup interaction ($F[57, 2603] = 15.96, p < 0.001$) and confirmed that offspring exposed to prenatal EtOH+NIC had shorter body lengths than all other groups and that controls (Air+VEH) and offspring exposed to prenatal alcohol alone (EtOH+VEH) had shorter body lengths than offspring exposed to prenatal nicotine alone (Air+NIC; SNK: $p < 0.05$, *figure 7*).

These results suggests that prenatal exposure to nicotine alone and combined prenatal exposure to EtOH and NIC altered the trajectory of offspring body length growth. Specifically, combined prenatal exposure to EtOH and NIC decreased body length growth while prenatal exposure to nicotine alone increased body length growth trajectory.

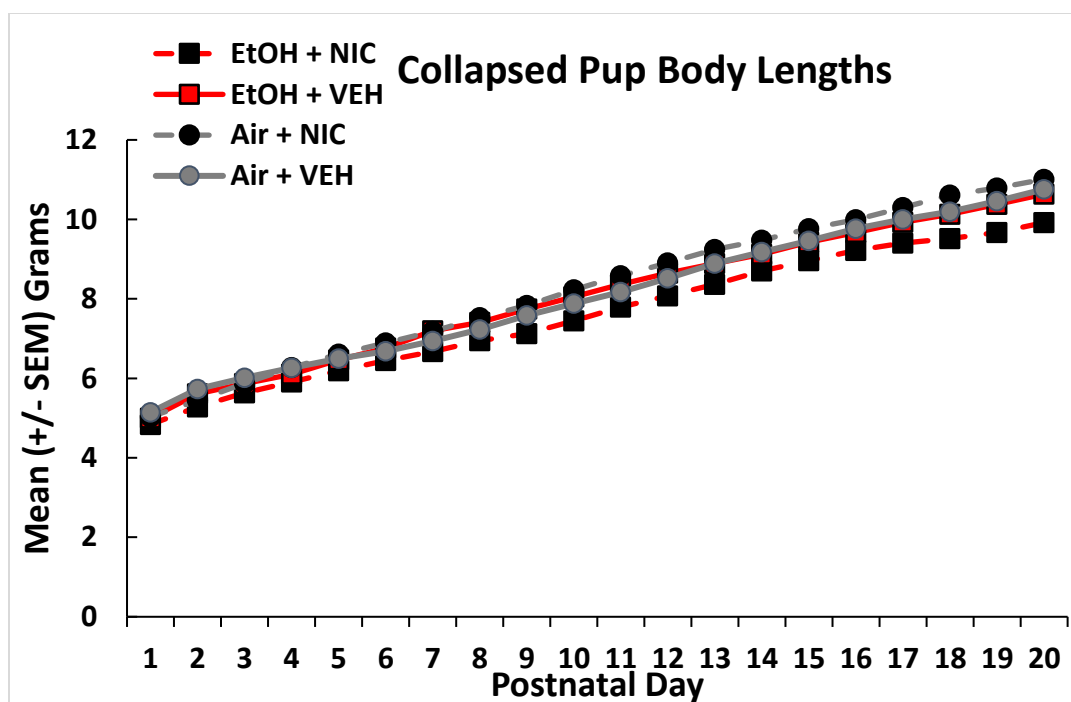


Figure 7: Offspring with combined prenatal exposure (EtOH+NIC) were significantly shorter in length compared to all other prenatal exposure groups. EtOH+NIC < EtOH+VEH, Air+VEH < Air+NIC, $F[57, 2603] = 15.96$, $p < 0.001$, SNK: $p < 0.05$.

Offspring Developmental Milestones

Once born, offspring were monitored for developmental milestones such as ear unfurling, teeth eruption, and eye opening. Due to the narrow range of when these milestones occur, these data were not normally distributed (Shapiro-Wilk's $p < 0.001$). Therefore, all milestones were analyzed using non-parametric measures. A Mann-Whitney U was used to separately examine main effects of Sex (Female, Male), EtOH (EtOH, Air), and NIC (NIC, VEH), while a Kruskal-Wallis test was used to examine overall prenatal exposure group effects (EtOH+NIC, EtOH+VEH, Air+NIC, Air+VEH).

Analyses on offspring ear unfurling occurrence indicated a main effect of EtOH with any subjects exposed to alcohol (EtOH+NIC, EtOH+VEH) experiencing significant delays in ear unfurling ($U = 1923, p = 0.002$) (*figure 8*). A follow-up Kruskal-Wallis analysis comparing all prenatal exposure groups confirmed this main effect ($K = 10.87, p = 0.012$). A similar effect was observed in eye opening occurrence, where analyses indicated a main effect of prenatal EtOH exposure on offspring eye opening ($U = 1740, p < 0.001$) where subjects exposed to any alcohol (EtOH+NIC, EtOH+VEH) experienced delayed eye opening compared to offspring not exposed to alcohol (Air+NIC, Air+VEH) (*figure 8*). A follow-up Kruskal-Wallis analysis examining all prenatal exposure groups confirmed this main effect of alcohol ($K = 17.35, p < 0.001$).

Analyses on offspring teeth eruption occurrence indicated a difference effect, with a potential interactive effect of combined prenatal exposure (EtOH+NIC) via main effects of both prenatal EtOH and NIC exposure separately. Therefore, a Kruskal-Wallis analysis was utilized to further examine this interaction. The Kruskal-Wallis analysis indicated an interactive effect of combined prenatal exposure (EtOH+NIC) on offspring teeth eruption, where subjects with combined prenatal exposure experienced significant delays in teeth eruption ($K = 39.95, p < 0.001$) (*figure 8*). A follow-up Chi-Square analyses confirmed that subjects with combined exposure had significant delays in teeth eruption compared to all other groups (1(EtOH+NIC) x 2 (EtOH+VEH): ($X^2 = 15.76, p = 0.008$), 1 (EtOH+NIC) x 3 (Air+NIC): ($X^2 = 28.65, p < 0.001$), 1 (EtOH+NIC) x 4 (Air+VEH): ($X^2 = 29.39, p < 0.001$).

Taking all milestone outcome together (ear unfurling, teeth eruption, eye opening), prenatal EtOH and combined exposure delayed both ear unfurling and eye opening; however, combined prenatal exposure of EtOH and NIC alone delayed teeth eruption. These findings indicate that prenatal alcohol exposure can delay all milestone occurrences, with combined

exposure specifically altering the trajectory of teeth eruption suggesting nicotine further exacerbating this effect.

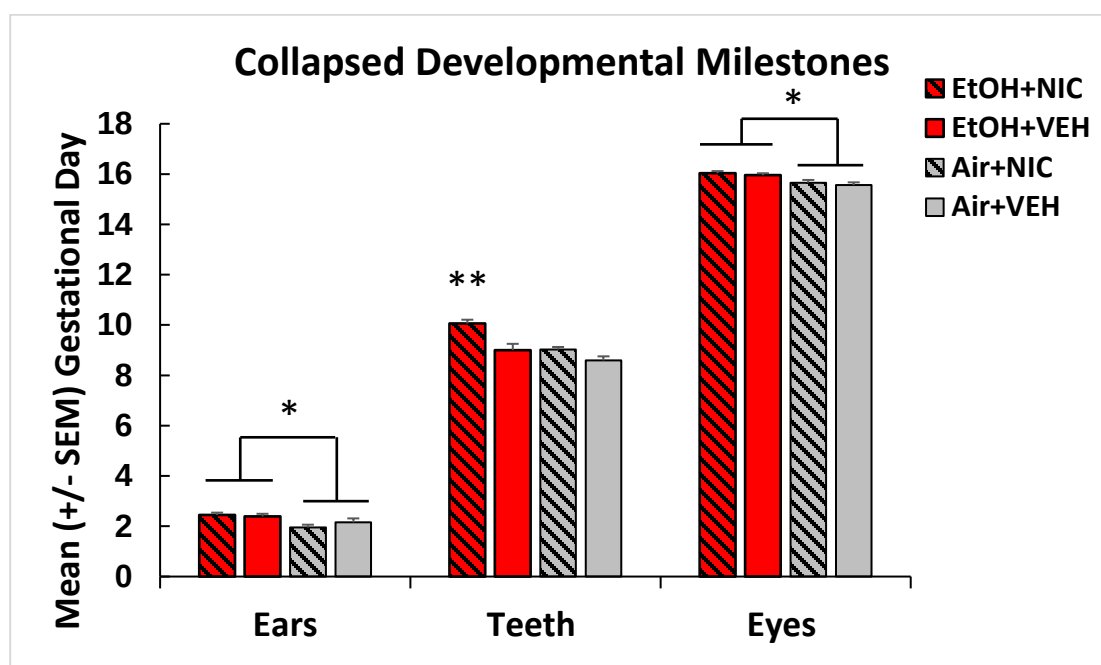


Figure 8: Offspring with any prenatal alcohol exposure (EtOH+NIC, EtOH+VEH) experienced delayed ear unfurling and eye opening compared to offspring with no prenatal alcohol exposure (Air+NIC, Air+VEH). Offspring with combined prenatal exposure (EtOH+NIC) experienced delayed teeth eruption compared to all other groups. *EtOH > Air, $p < 0.05$, **EtOH+NIC > EtOH+VEH, Air+NIC, Air+VEH, $p < 0.05$.

Group 1-2: Self-Administration

Analyses included a total of 57 subjects examined for their drug-seeking behavior. A total of 28 subjects (EtOH+NIC: 6 [2 females, 4 males], EtOH+VEH: 6 [3 females, 3 males], Air+NIC: 9 [4 females, 5 males], Air+VEH: 7 [3 females, 4 males]) self-administered nicotine (group 1), and a total of 29 subjects (EtOH+NIC: 8 [4 females, 4 males], EtOH+VEH: 6 [3

females, 3 males], Air+NIC: 8 [4 females, 4 males], Air+VEH: 7 [4 females, 3 males] self-administered the e-cigarette vehicle (group 2). The programmed outlier procedure through IBM SPSS was executed to detect any extreme outliers, and a total of 6 subjects were removed from analyses for meeting extreme outlier criteria, including 3 subjects that self-administered nicotine (group 1; EtOH+NIC: 2 females, Air+VEH: 1 female) and 3 subjects that self-administered vehicle (group 2; Air+NIC: 2 [1 female, 1 male], Air+VEH: 1 male).

Offspring drug-seeking behavior was examined using a self-administration paradigm where subjects utilized a nose poke hole function to self-administer (SAM) either nicotine (SAM NIC, group 1) or vehicle (SAM VEH, group 2). Testing lasted for 15 consecutive days, in one 1-hour session per day. During behavioral testing, a control table automatically recorded the number of valid pokes that trigger the vapor, total pokes, and total number of vapes delivered per session.

Self-Administration: Body Weight

Subject's body weight, food intake, and water intake was measured starting two days prior to the start of testing (PD28) and continues throughout the end of the paradigm (PD30-44). Offspring body weight was analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) x 2 (SAM NIC, SAM VEH) repeated measures ANOVA for PD 28-44. Results indicated a 2-way interaction of EtOH*NIC, main effect of sex, a main effect of alcohol, and a main effect of nicotine ($ps < 0.05$).

Follow-up analyses initially split data by prenatal alcohol exposure (EtOH, Air) and indicated a main effect of prenatal nicotine exposure within prenatal EtOH-exposed offspring, where offspring with combined prenatal exposure (EtOH+NIC) generally weighed less compared

to offspring with alcohol exposure only (EtOH+VEH) $F[1,18] = 11.96, p = 0.003$). Due to the interactions of EtOH*NIC, additional analyses used a 4 (EtOH+NIC, EtOH+VEH, Air+NIC, Air+VEH) x 2 (Female, Male) repeated measures ANOVA across PD 28-44 and yielded a Day*ExposureGroup interaction ($F[48,656] = 4.37, p < 0.001$), confirming that subjects exposed to prenatal EtOH+NIC weighed less than all other exposure groups (EtOH+VEH, Air+NIC, Air+VEH; SNK: $p < 0.05$). Importantly, there were no significant differences in overall offspring body weight between groups 1 and 2 (SAM NIC, SAM VEH).

However, there were additional interactions with Day, including a 2-way interaction of Day*Sex, a 2-way interaction of Day*EtOH, a 2-way interaction of Day*SAM, a 3-way interaction of Day*Sex*EtOH, a 3-way interaction of Day*Sex*NIC, and a 3-way interaction of Day*EtOH*SAM ($ps < 0.05$). Multiple follow-up analyses were utilized to examine body weights on individual days to further investigate these interactions by day. In short, sex differences were observed during PD 33-44 only ($ps < 0.05$), which coincides with the likely onset of puberty (around PD 30 for females, around PD 40 for males). Follow-up analyses on individual days further confirmed that female (*figure 9A*) and male (*figure 9B*) subjects with combined prenatal exposure (EtOH+NIC) weighed significantly less compared to all other exposure groups (EtOH+VEH, Air+NIC, Air+VEH), regardless of sex on PD 28-39 ($ps < 0.05$, SNK: $p < 0.05$).

Taken together, males gradually weighed more compared to females towards the end of the paradigm due to the onset of puberty and the increase of hormones. However, regardless of sex, combined prenatal exposure to EtOH and NIC caused less body weight growth throughout the SAM paradigm compared to individual prenatal exposure.

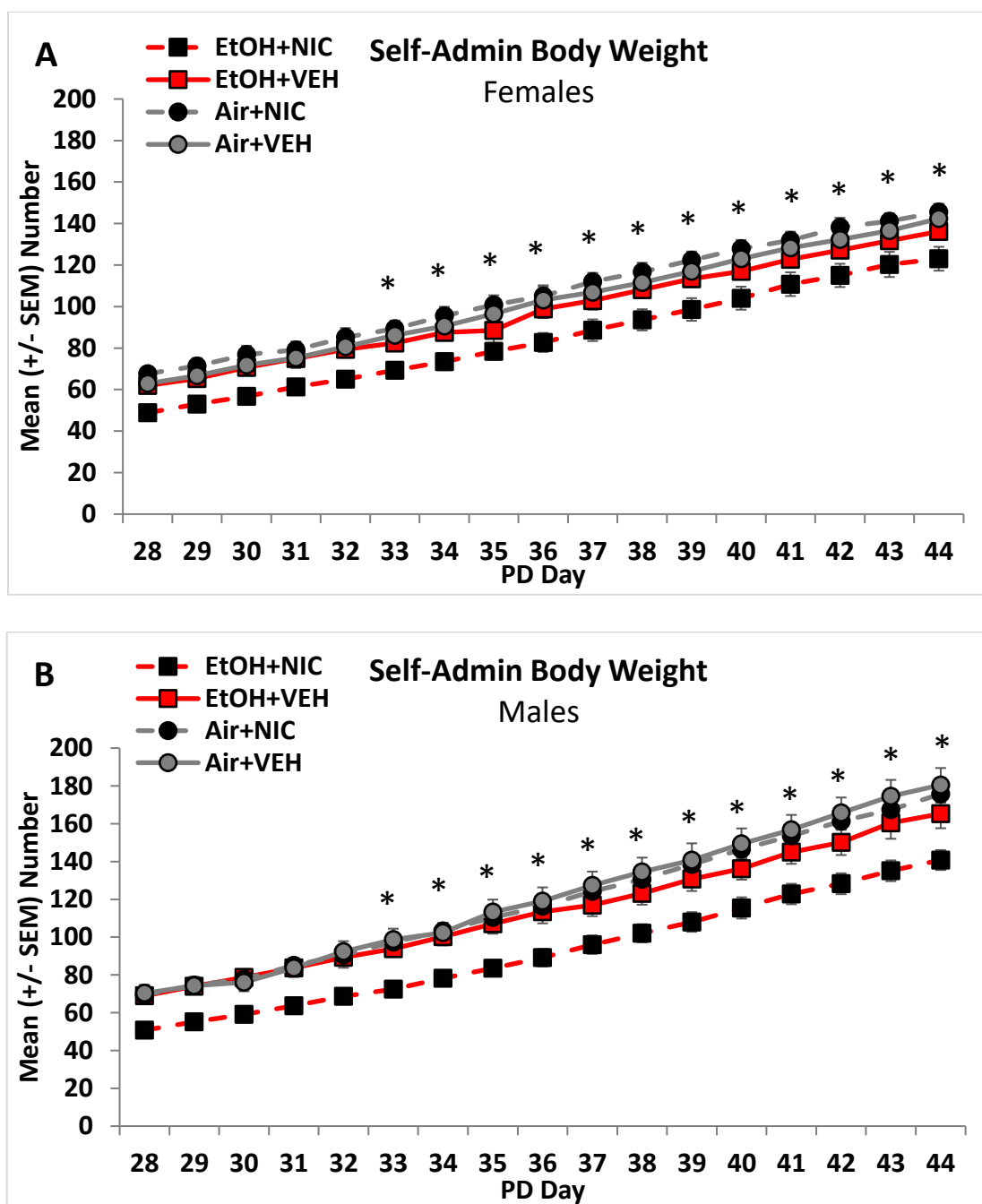


Figure 9: Male (A) and female (B) subjects with combined prenatal exposure (EtOH+NIC) weighed significantly less compared to all other prenatal exposure groups across all days, $F[48,656] = 4.37, p < 0.001$, SNK: $p < 0.05$. *Females < Males, $ps < 0.05$.

Self-Administration: Food Intake

Self-administration subjects' food intake was analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) x 2 (SAM NIC, SAM VEH) repeated measures ANOVA for PD 28-44. There was a main effect of sex ($F[1,41] = 8.06, p = 0.007$), where males had a higher food intake compared to females. In addition, there was a 2-way interaction of Day*Sex, a 2-way interaction of Day*EtOH, and a 3-way interaction of Day*EtOH*NIC ($ps < 0.05$).

Follow-up analyses initially split all data by sex (Females, Males). No main effects of prenatal EtOH or NIC exposure were observed within male subjects (*figure 10B*); however, a main effect of prenatal nicotine exposure within females ($F[1,20] = 10.33, p = 0.004$) indicated that females with prenatal nicotine exposure (EtOH+NIC, Air+NIC) had a higher food intake compared to females with no prenatal nicotine exposure (EtOH+VEH, Air+VEH; *figure 10A*). More specifically, this effect was observed on PD 28, throughout PD 34-37, and on PD 39 ($ps < 0.05$).

Taking these data together, prenatal exposure to NIC alone or the combination of EtOH and NIC increased offspring appetite with this effect observed within females only on PD 28, PD 34-37, and on PD 39. Excluding PD 28, this effect was mainly observed mid-way through the SAM paradigm; however, since no differences were observed between groups 1 and 2 all food intake differences are caused by prenatal exposure and not acute exposure during the paradigm. Furthermore, the differences in food intake does not directly mirror body weight differences, thus all changed reported for body weight during the SAM paradigm are not due to acute food intake.

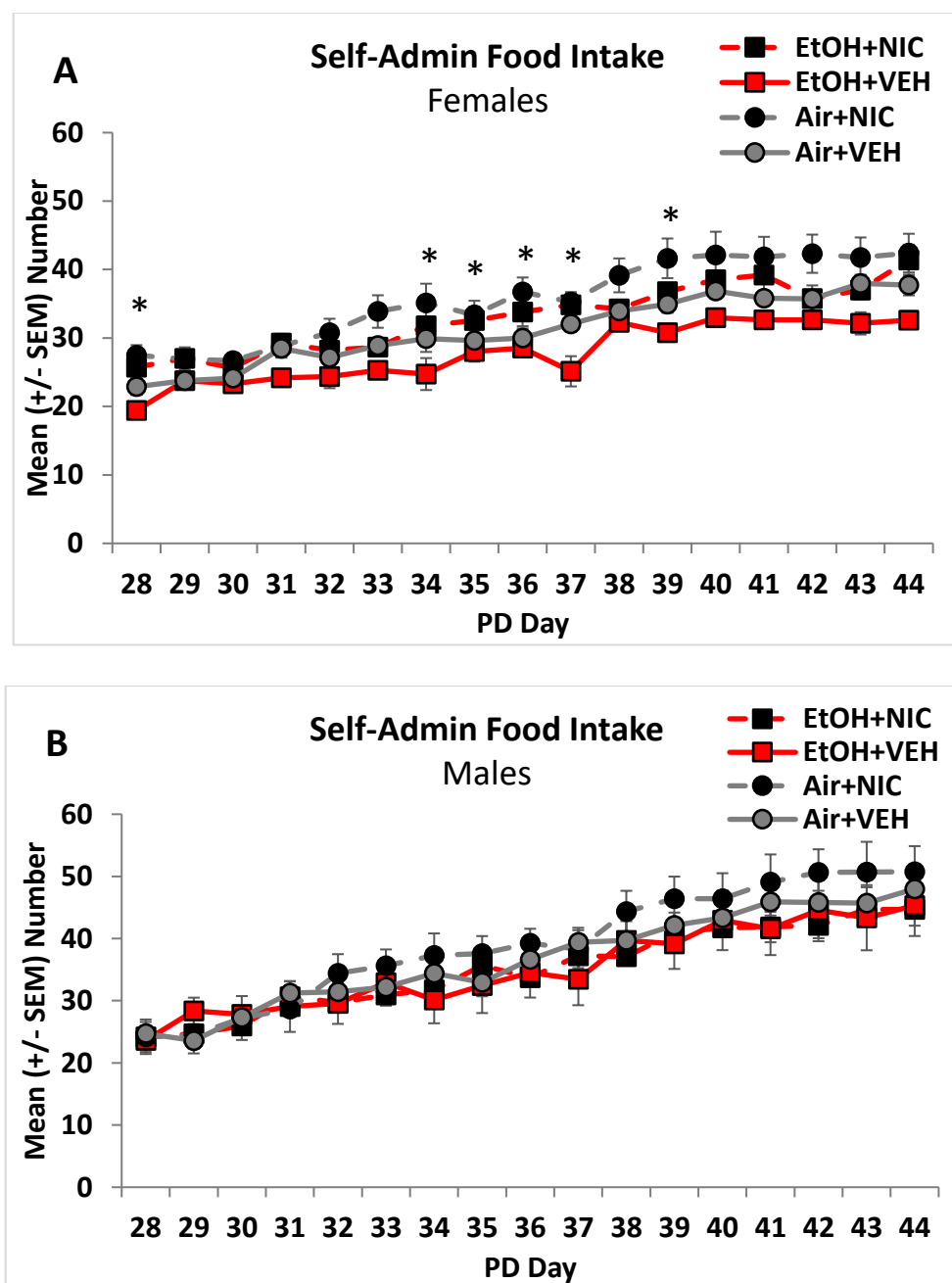


Figure 10: Overall, male (B) offspring ate more than female (A) offspring regardless of prenatal exposure or self-administration drug, $F[1,41] = 8.06$, $p = 0.007$. Females with any prenatal nicotine exposure (EtOH+NIC, Air+NIC) ate more compared to females with no prenatal nicotine exposure (EtOH+VEH, Air+VEH; A). *NIC > VEH, $ps < 0.05$.

Self-Administration: Water Intake

Subjects water intake during the SAM paradigm was analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) x 2 (SAM NIC, SAM VEH) repeated measures ANOVA for PD 28-44. Analyses indicated a main effect of alcohol on water intake ($F[1,41] = 12.46, p = 0.001$) with subjects with any type of prenatal alcohol exposure (EtOH+NIC, EtOH+VEH) drank significantly less water compared to subjects with no prenatal alcohol exposure (Air+NIC, Air+VEH). In addition, a 2-way interaction of Day*Sex, a 2-way interaction of Day*EtOH, and a 3-way interaction of Day*EtOH*NIC ($ps < 0.05$) were observed.

Follow-up analyses examining individual days were utilized to further examine these interactions, which further confirmed this overall effect of EtOH where subjects exposed to any prenatal alcohol (EtOH+NIC, EtOH+VEH) drank less water than their Air-exposed counterparts (Air+NIC, Air+VEH) throughout the SAM paradigm ($ps < 0.05$, *figure 11*) which did not vary by sex. These data suggests that overall, prenatal exposure to EtOH alone and the combined prenatal exposure of EtOH and NIC altered offspring water intake later in life. More specifically, it indicated they drank less water compared to offspring exposed to NIC alone and the control condition. Importantly, water intake did not vary between groups 1 and 2 within any exposure group, suggesting that any differences in water intake were also due to prenatal drug exposure rather than acute drug exposure. Furthermore, the differences in body weight do not directly mirror those in water-intake, and thus all changes reported for body weight during the SAM paradigm are not due to acute water intake.

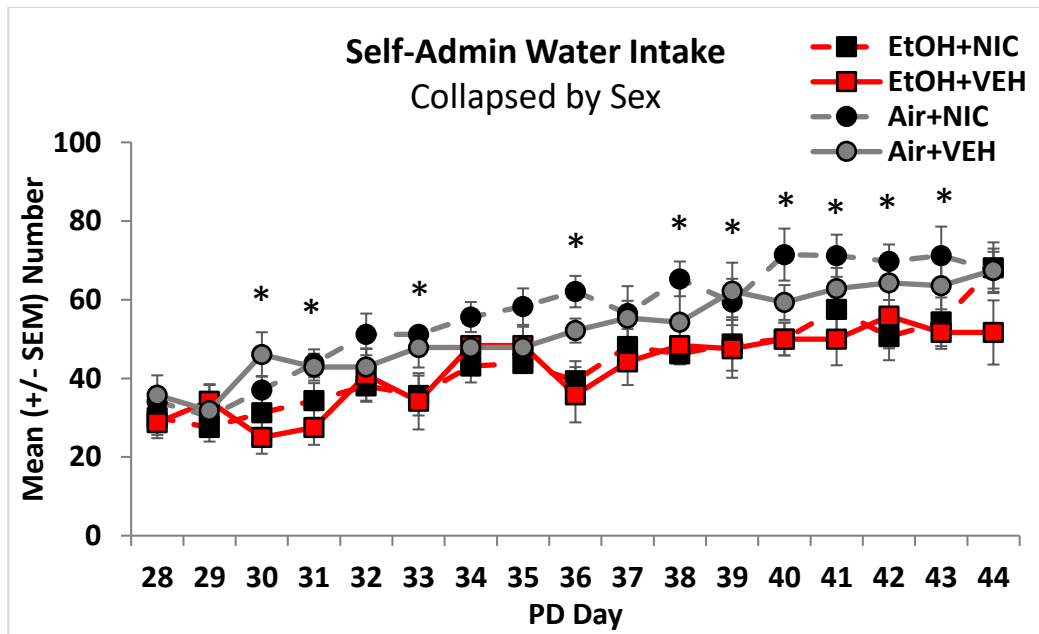


Figure 11: Offspring with any prenatal alcohol exposure (EtOH+NIC, EtOH+VEH) drank less compared to offspring with no prenatal alcohol exposure (Air+NIC, Air+VEH) $F[1,41] = 12.46$,

$$p = 0.001. *EtOH < Air, ps = 0.001.$$

Self-Administration: Total Pokes

The total number of nose pokes during the SAM paradigm indicates the severity of drug-seeking behaviors. Offspring total number of pokes during SAM was analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) x 2 (SAM NIC, SAM VEH) repeated measures ANOVA for all self-administration days, PD 30-44. Analyses indicated a main effect of self-administration drug ($F[1,37] = 49.00, p < 0.001$) where subjects that self-administered vehicle (SAM VEH, group 2; *figures 12A and 12B*) interacted with the nose poke hole more frequently compared to subjects that self-administered nicotine (SAM NIC, group 1; *figures 12C and 12D*). Additionally, analyses indicated an overall main effect of sex ($F[1,37] = 6.37, p = 0.016$) where females (*figures 12A and 12C*) interacted with the nose poke hole more frequently compared to

their male counterparts (*figures 12B and 12D*). All data was separated by self-administration drug group (SAM NIC, SAM VEH) to further investigate these effects.

Female subjects in group 1 (SAM NIC; *figure 12C*) had a significantly greater number of total pokes than males in the SAM NIC group ($F[1,18] = 4.67, p = 0.044$; *figure 12D*), suggesting that females self-administering nicotine exhibited more drug-seeking behavior compared to their male counterparts. All data was further separated by sex; however, no further main effects or interactions of prenatal EtOH or prenatal NIC exposure were observed in either group 1 (SAM NIC) or group 2 (SAM VEH). This suggests that overall, subjects that self-administered vehicle (SAM VEH) interacted with the nose poke hole more frequently compared to subjects that self-administered nicotine (SAM NIC), and females that self-administered nicotine (SAM NIC) did so more than males. Importantly, no differences were observed between days or in prenatal exposure group suggesting these effects are mainly sex-dependent.

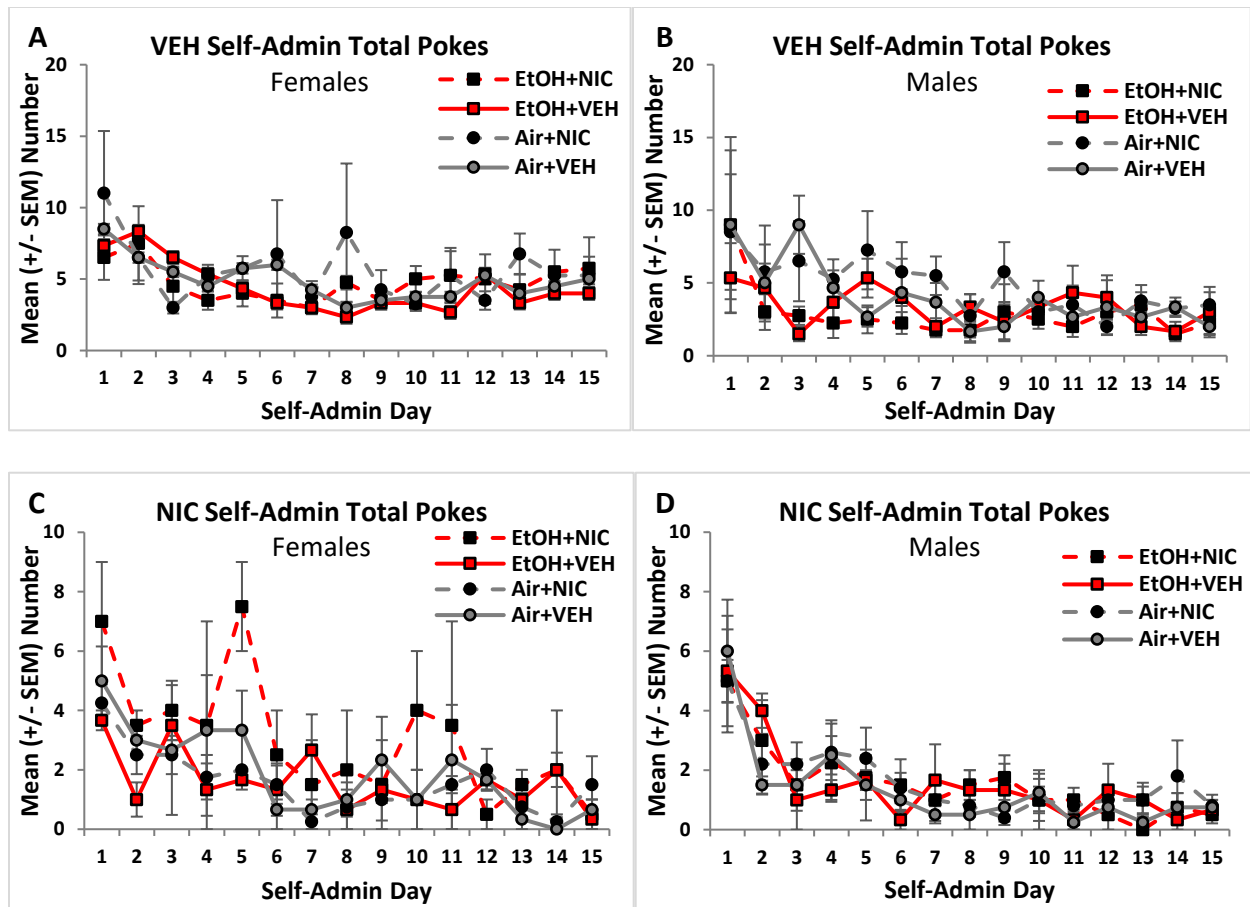


Figure 12: Overall, subjects had greater total pokes for the vehicle (A and B) compared to nicotine (C and D). In addition, females (A and C) interacted with the nose poke hole more compared to males (B and D) across all days with no differences in prenatal exposure. Moreover, females self-administering nicotine (C) interacting with the nose poke hole more compared to males self-administering nicotine (D). Female SAM NIC > Male SAM NIC, $F[1,37] = 6.37$, $p = 0.016$. NIC < VEH, $F[1,37] = 49.00$, $p < 0.001$.

Self-Administration: Total Vapes

Since there was an enforced timeout of 10 seconds between vapes during SAM, the total vapes indicates how many vapes each subject actually received (in contrast to total pokes, which

is the total attempts to receive vape). The total number of vapes delivered to subjects was analyzed using a 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) x 2 (SAM NIC, SAM VEH) repeated measures ANOVA for all self-administration days (PD 30-44). Analyses indicated a main effect of self-administration drug group ($F[1,37] = 46.60, p < 0.001$) where subjects self-administered vehicle (SAM VEH, group 2; *figures 13A and 13B*) significantly more compared than nicotine (SAM NIC, group 1; *figures 13C and 13D*). In addition, there was a main effect of sex ($F[1,37] = 7.99, p = 0.008$) where females (*figures 13A and 13C*) successfully self-administered more vapes compared to males overall (*figures 13B and 13D*). All data was separated by self-administration drug group (SAM NIC, SAM VEH) to further investigate this main effect.

Follow-up analyses indicated that female subjects self-administering NIC (SAM NIC, group 1; *figure 13C*) successfully received significantly more nicotine vapor than males ($F[1,18] = 5.61, p = 0.029$; *figure 13D*). A similar effect of sex ($F[1,19] = 4.20, p = 0.054$) was trending within subjects self-administering vehicle (SAM VEH, group 2; *figures 13A and 13B*) which further supports that female subjects overall received more vapor compared to males, regardless of self-administration drug. Taken together, these data suggests that overall, subjects self-administering vehicle exhibited more drug-seeking behavior compared to subjects self-administering nicotine. Female subjects received significantly more vapor in both groups, suggesting that females exhibited more severe drug compared to males regardless of self-administration drug. Importantly, no differences were observed in day or prenatal exposure group suggesting these effects are mainly sex-dependent with prenatal drug exposure not influencing these behaviors.

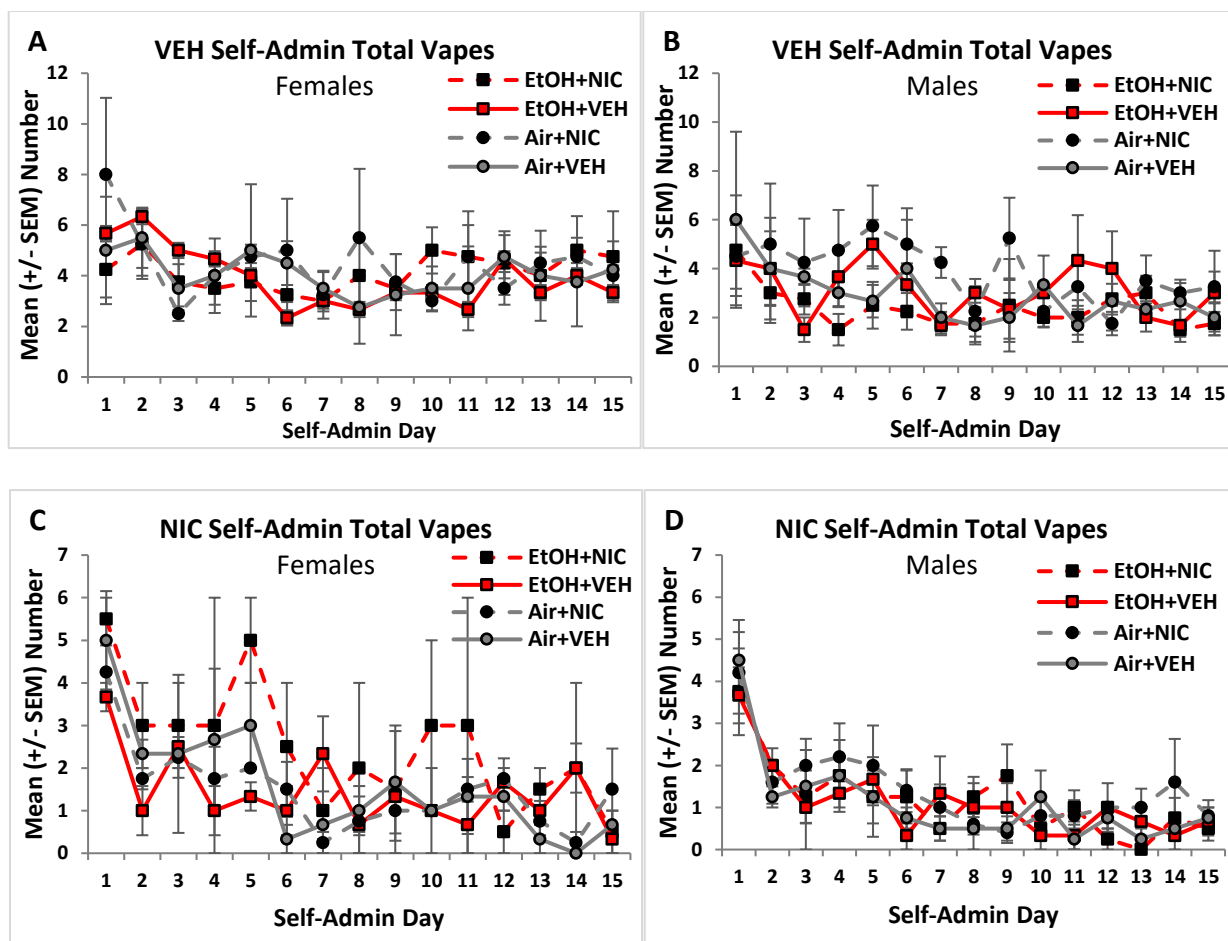


Figure 13: Overall, females (A and C) received more vapor compared to males (B and D) regardless of prenatal exposure or self-administration drug across all days. Vehicle (A and B) was self-administered more compared to nicotine (C and D) across all days, regardless of sex. Female SAM > Male SAM, $F[1,37] = 7.99$, $p = 0.008$. NIC < VEH, $F[1,37] = 46.60$, $p < 0.001$.

Group 3-4: Novelty Object Recognition

A total of 65 subjects were examined for their memory performance, with 33 tested during adolescence (EtOH+NIC: 9 [4 females, 5 males], EtOH+VEH: 6 [3 females, 3 males], Air+NIC: 10 [5 females, 5 males], Air+VEH: 8 [4 females, 4 males]) and 32 tested during

adulthood (EtOH+NIC: 9 [5 females, 4 males], EtOH+VEH: 5 [3 females, 2 males], Air+NIC: 10 [4 females, 6 males], Air+VEH: 8 [4 females, 4 males]).

Offspring memory performance was evaluated through a novelty object recognition (NOR) task that lasted 2 consecutive days, with 10-minute sessions each day. On the first day of testing, two identical objects were placed in a 20x20 plexiglass box for the subject to freely explore both objects with no limitations. On the second day of testing, one of the identical objects from the previous day was replaced with a new novelty object and the subjects freely explored both the old and new object with no limitations. A camera recorded each session, that was later coded via ODLog for their total interaction time and frequency of behaviors. Two individuals separately coded each video; therefore, an inner rate reliability analysis was utilized to confirm data accuracy (Cronbach's Alpha = 88%). The discriminatory index for interaction time with the novelty object versus the old object (novelty object time / total novel object and familiar object time) was calculated for each subject.

Group 3: Early Adolescence

A 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) ANOVA indicated a 2-way interaction of EtOH*NIC ($F(1,25) = 5.58, p = 0.026$) on subject's discriminatory index. Follow-up analyses split all data by prenatal alcohol exposure (EtOH, Air). Among adolescent subjects exposed to any prenatal alcohol (EtOH+NIC, EtOH+VEH), subjects with prenatal alcohol exposure alone (EtOH+VEH) had a significantly lower discriminatory index compared to subjects with combined prenatal exposure (EtOH+NIC) ($F(1,13) = 7.87, p = 0.015$) (*figure 16*). This suggests that adolescent offspring prenatally exposed to alcohol alone exhibited more discrimination towards the objects compared to all other exposure groups. Follow-up analyses

separating all data by nicotine exposure (NIC, VEH) elicited a similar effect that was near significance, where subjects exposed to nicotine alone (Air+NIC) had a lower discriminatory index compared to subjects with combined prenatal exposure (EtOH+NIC) ($F[1,17] = 4.40, p = 0.051$). A 4 (EtOH+NIC, EtOH+VEH, Air+NIC, Air+VEH) x 2 (females, males) ANOVA was also run to further examine the interaction of EtOH*NIC, however, no overall effect of prenatal exposure group was observed.

Additional analyses were completed to more thoroughly examine the differences observed in the discriminatory index scores, including interaction times with the familiar (old) versus novel objects. A 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) repeated measures ANOVA for interaction time for each individual object yielded an Object*EtOH*NIC interaction ($F[1,25] = 5.15, p = 0.032$). A follow-up 4 (EtOH+NIC, EtOH+VEH, Air+NC, Air+VEH) x 2 (Female, Male) analyses for each object to further examine this interaction indicated that subjects prenatally exposed to alcohol alone (EtOH+VEH) interacted with the familiar (old) object significantly more than adolescent offspring exposed to combined EtOH+NIC or the control condition (Air+VEH) ($F[3,25] = 3.40, p = 0.03$, SNK: $p < 0.05$, *figure 14*). This effect was also seen in the total interaction time, and no differences in prenatal alcohol or prenatal nicotine exposure were observed in interaction time with the novel object. These results suggests that subjects exposed to prenatal alcohol exhibited more memory impairments compared to all other groups.

Taken together, this suggests that adolescent offspring exposed to prenatal alcohol alone (EtOH+VEH) may impair learning and memory performance as they showed significantly more interest in the old object versus the novelty object. Additionally, they were overall more active which may have influenced these effects. Adolescent offspring with prenatal nicotine exposure

alone (Air+NIC) showed a similar trend in memory performance and activity levels, but was not statistically significant.

Group 4: Adulthood

A 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) ANOVA examining adult subject's discriminatory indexes indicated a 2-way interaction of EtOH*NIC ($F[1,24] = 4.56, p = 0.043$). Follow-up analyses separating all data by alcohol exposure (EtOH, Air) indicated a main effect of nicotine exposure ($F[1,14] = 6.92, p = 0.020$) on subjects with no prenatal alcohol exposure, suggesting that subjects prenatally exposed to nicotine alone (Air+NIC) had a significantly smaller discriminatory index compared to subjects in the control condition (Air+VEH) (*figure 16*). This suggests that adult offspring prenatally exposed to nicotine alone exhibited more discrimination towards the objects compared to all other prenatal exposure groups. No further main effects or interactions were observed in prenatal alcohol exposure, and no main effects were observed in either alcohol or nicotine when data was split by nicotine exposure (NIC, VEH).

To further examine the differences observed in the discriminatory index scores, a 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) repeated measures ANOVA examining old (familiar) versus novelty object interaction time was run. This analysis indicated no main effects or interactions of prenatal EtOH or prenatal NIC exposure by day; however, an overall main effect of prenatal NIC exposure ($F[1,24] = 6.13, p = 0.021$) was observed, where subjects with any prenatal nicotine exposure (EtOH+NIC, Air+NIC) were more active and interacted more with the objects compared to subjects with no prenatal nicotine exposure (EtOH+VEH, Air+VEH) (*figure 15*). This was confirmed in examining total interaction time, where subjects

with any prenatal nicotine exposure (EtOH+NIC, Air+NIC) were more active compared to subjects with no prenatal nicotine exposure (EtOH+VEH, Air+VEH) ($F[1,24] = 6.13, p = 0.021$).

Taken together, this suggests that adult offspring exposed to prenatal nicotine alone (Air+NIC) exhibited more discrimination towards the objects compared to all other exposure groups; however, no differences were observed in familiar versus novelty object interaction time. Adult offspring with any prenatal nicotine exposure (EtOH+NIC, Air+NIC) were additionally more active overall compared to offspring with no prenatal nicotine exposure (EtOH+VEH, Air+VEH) which may have contributed to this effect.

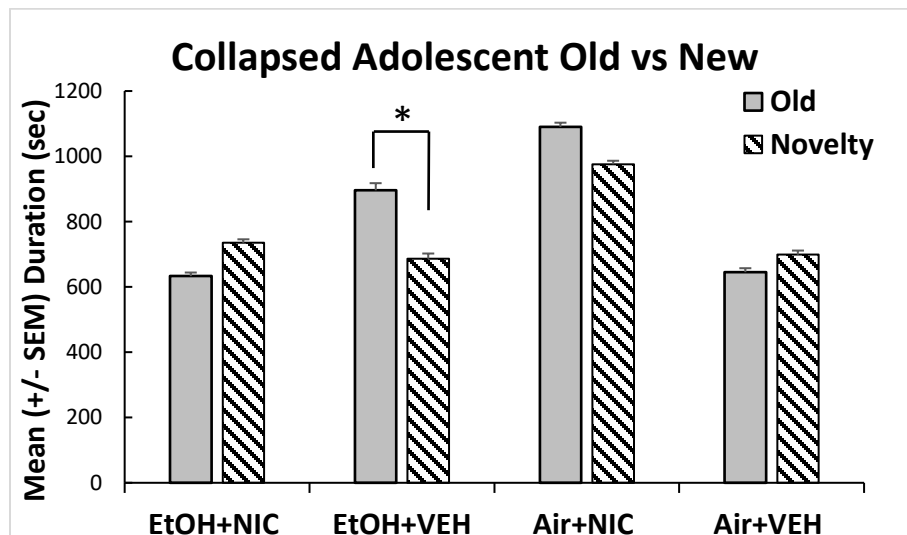


Figure 14: Offspring with prenatal exposure to alcohol alone (EtOH+VEH) interacted with the old object more compared to offspring with combined prenatal exposure (EtOH+NIC) and the control condition (Air+VEH). *Old > New, $F[3,25] = 3.40, p = 0.03$, SNK: $p < 0.05$.

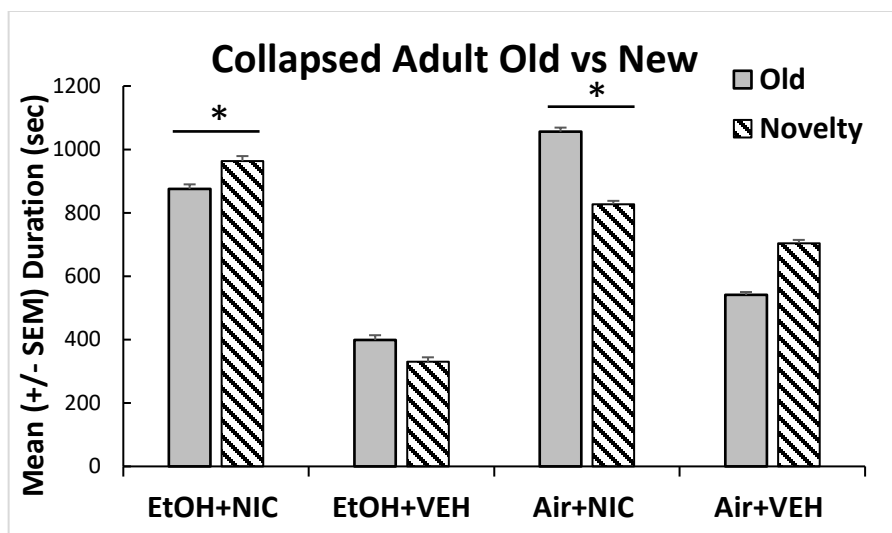


Figure 15: Offspring with any prenatal nicotine exposure (EtOH+NIC, Air+NIC) interacted more overall compared to offspring with no prenatal nicotine exposure (EtOH+VEH, Air+VEH), with no differences in old versus new object interaction time. *NIC > VEH, $F[1,24] = 6.13$, $p = 0.021$.

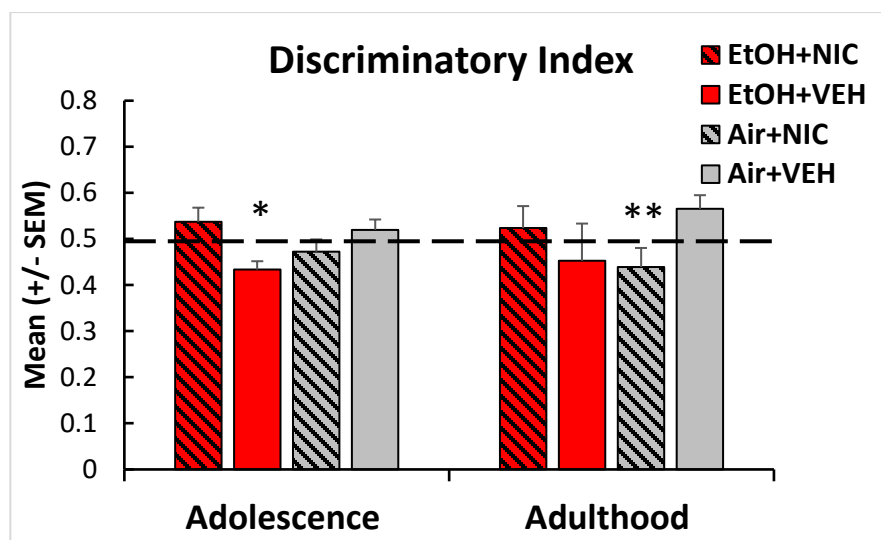


Figure 16: Adolescents with prenatal exposure to alcohol alone (EtOH+VEH) had a lower discriminatory index compared to adolescents with combined prenatal exposure and the control condition (EtOH+NIC, Air+VEH). Adults with prenatal exposure to nicotine alone (Air+NIC) had a lower discriminatory index compared to all other exposure groups. *EtOH+VEH <

EtOH+NIC, Air+NIC, Air+VEH, $F[1,13] = 7.87, p = 0.015$. **Air+NIC < EtOH+NIC, EtOH+VEH, Air+VEH, $F[1,14] = 6.92, p = 0.020$.

Offspring Tissue

Offspring tissue was collected once all behavioral tasks were completed within the litter (between PD 61-68). Each subject's body, brain, liver, heart, lung weights were recorded. All data were analyzed using 2 (EtOH, Air) x 2 (NIC, VEH) x 2 (Female, Male) ANOVAs.

Offspring Body Weight

Analyses of offspring's body weights indicated a main effect of sex, a main effect of alcohol, a 2-way interaction of EtOH*NIC, and a 3-way interaction of Sex*EtOH*NIC ($ps < 0.05$). All data was initially split by sex (Female, Male) to further investigate these interactions and main effects.

No main effects or interactions of prenatal EtOH or NIC exposure were observed within female subjects. However, a main effect of alcohol and a 2-way interaction of EtOH*NIC ($ps < 0.05$) was observed within male subjects. A follow-up 4-way (EtOH+NIC, EtOH+VEH, Air+NC, Air+VEH) ANOVA was conducted to further examine the EtOH*NIC interaction, and found that male subjects exposed to the combination of prenatal EtOH+NIC weighed significantly less than all other groups ($F[3, 68] = 11.26, p < 0.001$; SNK: $p < 0.05$) (figure 17). No significant differences were observed within female subjects, suggesting that prenatal drug exposure does not alter female body weight later in life; however, combined prenatal exposure (EtOH+NIC) altered male offspring body weight later in life.

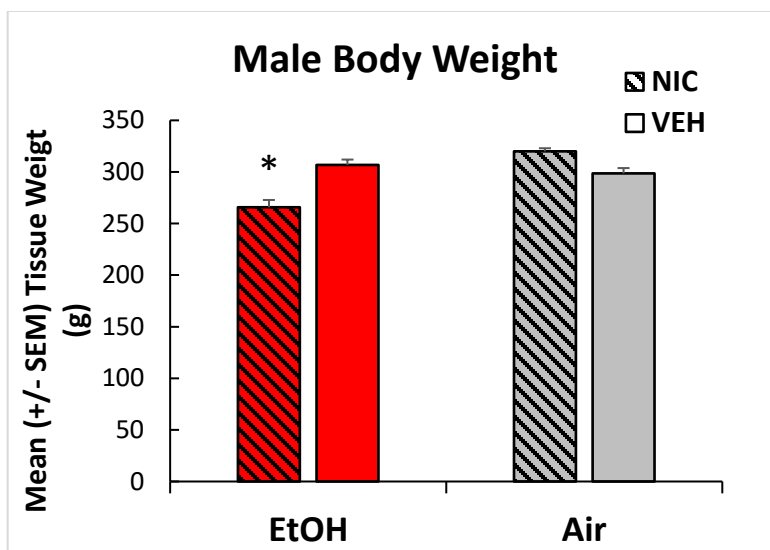


Figure 17: Males with combined prenatal exposure (EtOH+NIC) weighed significantly less compared to all other exposure groups. *EtOH+NIC < Air+VEH < EtOH+VEH, Air+NIC, $F[3, 68] = 11.26, p < 0.001$; SNK: $p < 0.05$.

Offspring Brain Weight

Similarly, analyses examining offspring brain volume indicated a main effect of sex, a main effect of alcohol exposure, and a 2-way interaction of EtOH*NIC ($ps < 0.05$). A follow-up 4-way (EtOH+NIC, EtOH+VEH, Air+NC, Air+VEH) ANOVA was conducted to further examine the EtOH*NIC interaction, and found that male subjects exposed to the combination of prenatal EtOH+NIC had significantly lower brain gross weights than all other groups, ($F[3, 68] = 8.83, p < 0.001$; SNK: $p < 0.05$) (figure 18). In contrast, females showed no differences among prenatal exposure groups.

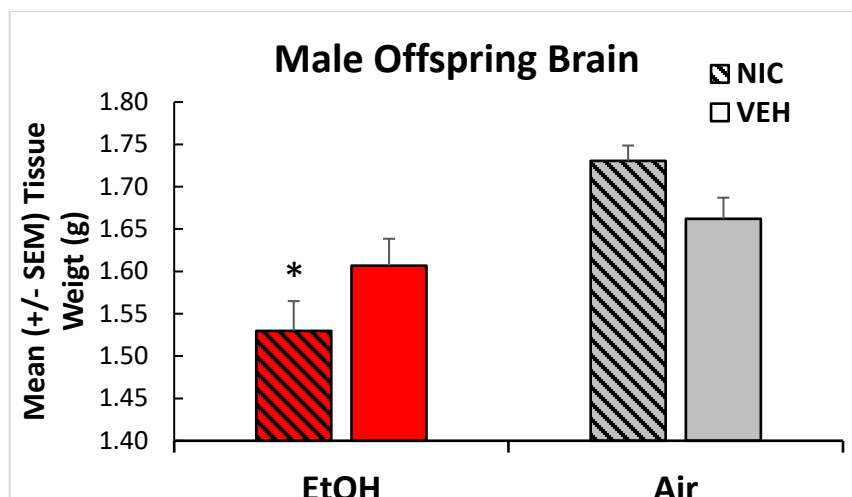


Figure 18: Male subjects with combined prenatal exposure had significantly lower brain gross weight compared to all other prenatal exposure groups. *EtOH+NIC < EtOH+VEH, Air+NIC, Air+VEH, $F[3, 68] = 8.83$, $p < 0.001$; SNK: $p < 0.05$.

Offspring Liver Weight

Analyses examining offspring liver weight indicated a main effect of sex, a main effect of alcohol, and a 3-way interaction of Sex*EtOH*NIC ($ps < 0.05$). All data was separated by sex (Female, Male) to further investigate these main effects and interactions. Follow-up analyses indicated a main effect of alcohol in both females and males, a main effect of nicotine in females only, and a 2-way interaction of EtOH*NIC in males only ($ps < 0.05$).

A follow-up 4-way (EtOH+NIC, EtOH+VEH, Air+NC, Air+VEH) ANOVA was conducted to further examine the EtOH*NIC interactions and main effects within each sex. Female subjects exposed to any prenatal nicotine (EtOH+NIC, Air+NIC) had significantly larger liver weights compared to subjects with no prenatal nicotine exposure (EtOH+VEH, Air+VEH) ($F[3,65] = 4.30$, $p = 0.008$, SNK: $p < 0.05$). In contrast, male subjects exposed to the combination of EtOH+NIC prenatally had significantly smaller liver weights than those exposed

to prenatal nicotine alone (Air+NIC) ($F[3,68] = 7.55, p < 0.001$) (figure 19). Thus, prenatal exposure to EtOH and/or NIC may have sex- and drug-specific effects on gross liver weight.

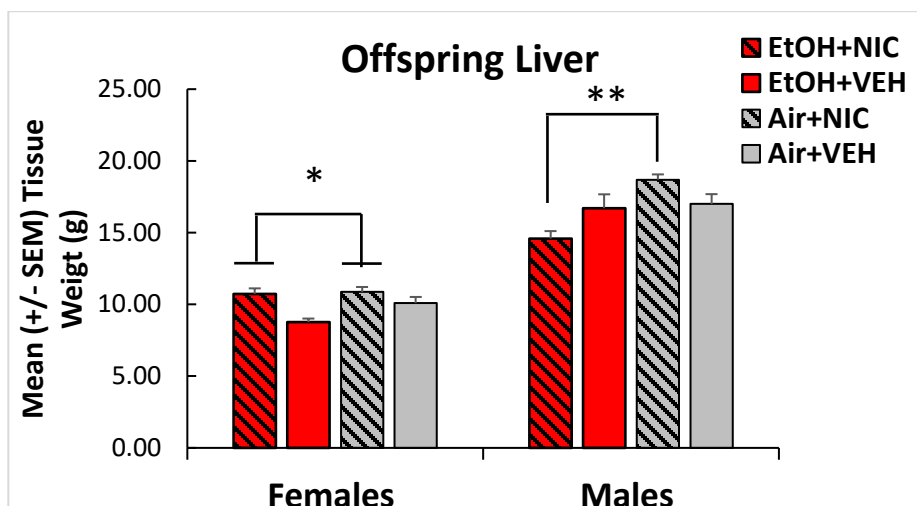


Figure 19: Female offspring with any prenatal nicotine exposure (EtOH+NIC, Air+NIC) had significantly larger liver weights compared to females prenatally exposed to alcohol alone (EtOH+VEH), *NIC > VEH, $F[3,65] = 4.30, p = 0.008$, SNK: $p < 0.05$. Male offspring prenatally exposed to nicotine alone (Air+NIC) had significantly larger livers compared to males with combined prenatal exposure (EtOH+NIC). **EtOH+NIC < EtOH+VEH, Air+VEH < Air+NIC, $F[3,68] = 7.55, p < 0.001$, SNK: $p < 0.05$.

Offspring Heart Weight

Analyses examining offspring heart volume indicated a main effect of sex ($F[1,109] = 10.46, p = 0.002$), where females had smaller heart volume compared to males, regardless of prenatal drug exposure (figure 20).

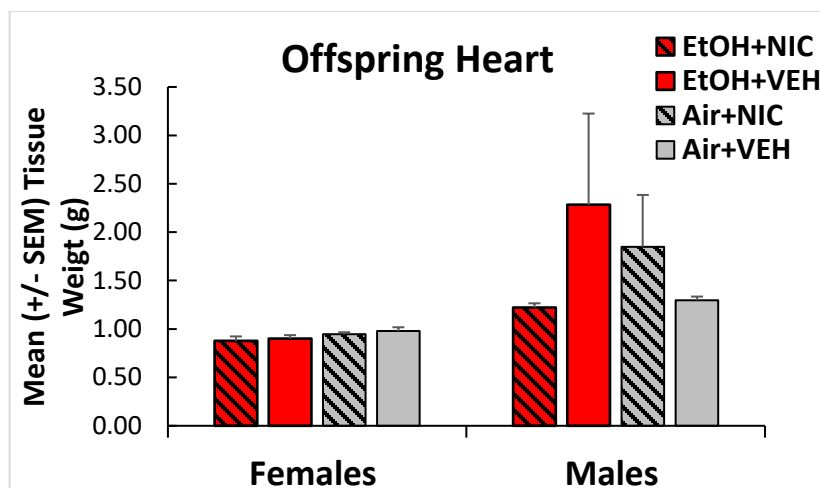


Figure 20: Overall, male offspring had significantly larger hearts compared to female offspring.

Females < Males, $F[1,109] = 10.46$, $p = 0.002$

Offspring Lung Weight

Analyses examining offspring lung volume indicated a main effect of sex, a main effect of alcohol, a main effect of nicotine, and a 2-way interaction of Sex*EtOH ($ps < 0.05$). All data was split by sex to further investigate these interactions and main effects. Follow-up analyses indicated a main effect of alcohol ($F[1,65] = 12.76$, $p < 0.001$) and a main effect of nicotine ($F[1,65] = 7.72$, $p = 0.007$) in female offspring only.

A follow-up 4-way (EtOH+NIC, EtOH+VEH, Air+NC, Air+VEH) ANOVA was conducted to further examine the EtOH*NIC interactions and main effects within female offspring. Female offspring prenatally exposed to any EtOH (EtOH+NIC, EtOH+VEH) had significantly smaller lung weights than those exposed to prenatal nicotine exposure alone (Air+NIC) ($F[3, 65] = 3.84$, $p = 0.014$, SNK: $p < 0.05$) (figure 21).

Taking all tissue weight data together, these data suggests that prenatal exposure to EtOH and/or NIC may have sex- and drug-specific effects on body, brain, liver, and lung weight but not heart weight.

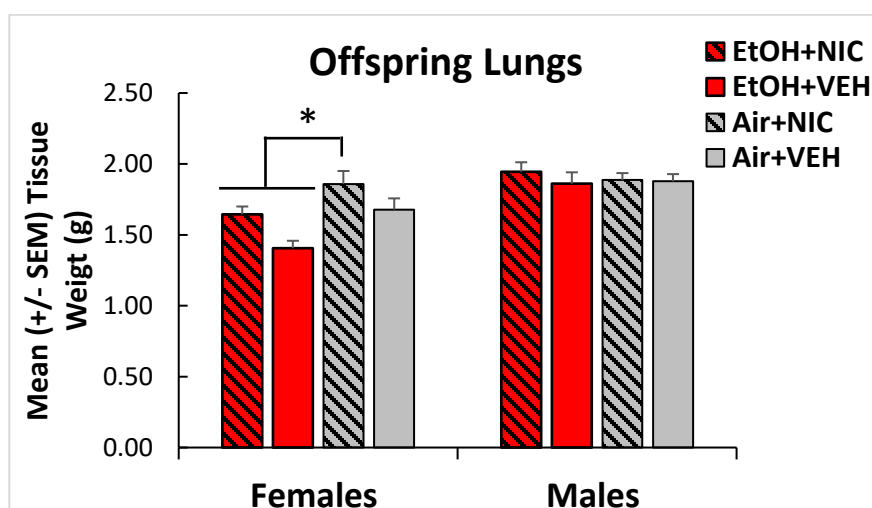


Figure 21: Females with any prenatal alcohol exposure (EtOH+NIC, EtOH+VEH) had significantly smaller lungs compared to females prenatally exposed to nicotine alone (Air+NIC).

No differences in prenatal exposure were observed in males. *EtOH+NIC, EtOH+VEH <

Air+VEH < Air+NIC, $F[3, 65] = 3.84$, $p = 0.014$, SNK: $p < 0.05$.

Discussion

This study examined whether combined prenatal exposure to alcohol and nicotine via e-cigarettes during the first trimester of pregnancy alters drug-seeking behavior during adolescence and memory performance during adolescence and adulthood.

Pregnant Sprague-Dawley dams were exposed to vaporized alcohol, nicotine via e-cigarettes, the combination, or the control vehicle for 3 hours once per day from gestational day (GD) 5-11, mimicking the first human trimester of pregnancy. Blood samples were collected on the first and last day of vapor inhalation drug exposure to confirm target drug levels and observe potential interactive pharmacokinetic effect of combined exposure to alcohol and nicotine. Dam body weights, food intake, and water intake were recorded from GD 0-22 to examine potential nutritional confounds during and after drug exposure. Offspring body weights, body lengths, and developmental milestones were recorded from birth till being weaned (postnatal day (PD) 1-20) to monitor development. Later in life, 2 sex pairs from each litter were examined using a self-administration paradigm during early adolescence (PD 30-44), novelty object recognition during adolescence (PD 30-31), and novelty object recognition during adulthood (PD 60-61).

Prenatal Drug Exposure

Dam body weight gain during the drug exposure (GD 5-11) did not differ, which alleviates some of the concerns of potential nutritional confounds during drug exposure. Dams exposed to any alcohol (EtOH+NIC, EtOH+VEH) during the first trimester had slower body weight gain later in pregnancy; however, dam food intake may have influenced some of this effect. Dams exposed to any type of nicotine (EtOH+NIC, Air+NIC) generally ate more food compared to all other groups later in pregnancy once drug exposure had ceased. Specifically, this

food intake difference was mainly observed leading up to differences in body weight gain, which may have contributed to NIC-exposed dams having greater body weight gain compared to dams exposed to alcohol. However, these results contradict each other as it applies to general EtOH or general NIC exposure, including combined exposure. This implies that nicotine exposure may have primarily driven the appetite increase within dams exposed to the combination, which was only partly reflected in dam body weight gain. Previous literature in clinical models have reported nicotine use as an appetite suppressor, with participants having a higher food intake during cessation as a coping mechanism for the negative effects of withdrawal (Anker et al., 2021) which is reflected in the current study.

There is extremely sparse clinical and preclinical research examining how alcohol cessation during pregnancy may decrease body weight gain; in this study, we can only infer that dams who were previously exposed to nicotine and had an increased appetite may have contributed to these group differences. No significant differences between exposure groups were observed in dam water intake, suggesting drug exposure does not influence water intake during drug exposure and later in pregnancy.

Dams' core body temperatures were measured pre- and post-vaporized drug exposure from GD 5-11. The core body temperatures recorded before the prenatal vapor paradigm exposure began (pre-temp on GD 5) were analyzed separately as a baseline temperature measure. There were no differences among prenatal exposure groups on baseline core body temperatures. However, once the paradigm began, dams exposed to any nicotine (EtOH+NIC, Air+NIC) had lower starting temperatures each day for the remainder of the paradigm, which suggests that nicotine exposure may alter thermoregulation. This finding has been previously observed in a similar prenatal exposure model of nicotine and THC (Breit et al., 2022). Furthermore, this

lowered starting temperature may have impacted the expected temperature changes during the prenatal vapor inhalation paradigm; dams exposed to any nicotine (EtOH+NIC, Air+NIC) had significantly less drastic temperature changes during each session.

Dam offspring outcomes was recorded after birth, including gestational day (GD) length, the number of pups born, the sex ratio of pups, and number of females born, and number of males born. Drug exposure did not influence any birth outcomes; however, a trending effect of nicotine alone (Air+NIC) was observed in the number of female offspring born. Given that the paternal parent determines biological sex, and the drug exposure occurred during the maternal parent's pregnancy, this difference was likely caused by random assignment of the male breeders.

Dam Tissue

Dam tissue was collected once pups were weaned from the mother (PD 21), where their body, brain, liver, heart, and lung weights were recorded. Analyses indicated that any type of nicotine exposure (EtOH+NIC, Air+NIC) resulted in larger brain weights compared to dams with no nicotine exposure (EtOH+VEH, Air+VEH). Since differences were only observed within groups exposed to any nicotine, it suggests that this effect was primarily carried by nicotine exposure with alcohol not influencing it. This is not consistent with previous literature, as previous research in clinical models reported a decrease in brain volume in individuals smoking nicotine (Chang et al., 2024). In addition, dam brain to body weight ratio was calculated to further examine this effect. Dams with any nicotine exposure had a significantly higher brain to body weight ratio, indicating they have a larger brain weight compared to their body weight. The study by Chang et al. (2024) included subjects' total body mass in analysis, suggesting that

subject brain volume results were independent of body weight. Moreover, there are differences in species, nicotine dosing, the timing of nicotine administration, and the routes of administration between the studies, and other research in this area is limited.

Developmental Milestones

Offspring with combined prenatal exposure (EtOH+NIC) during the first trimester overall weighed significantly less and were significantly shorter in length compared to all other prenatal exposure groups. These effects were more prominent during postnatal days (PD) 3-20. However, litters were culled on PD 1, and thus variations in initial litter sizes may have contributed to offspring weights and lengths during PD 1-2. As litters within the study became a consistent size, any prenatal drug exposure-related changes may have become more evident (by PD 3).

The offspring body weight differences observed in this study are consistent with previous research. Clinical models separately examining prenatal exposure of alcohol and nicotine have indicated low birth weight and smaller body size that may overcome with age (O’Keeffe et al., 2015, Reeves et al., 2008, White et al., 2022). On the contrary, preclinical models on individual prenatal drug exposure reported prenatal exposure to alcohol had long-lasting effects on offspring body weight, with this effect not seen in prenatal nicotine exposure (Breit et al., 2019, Breit et al. 2022). In the current study, subjects with combined prenatal exposure (EtOH+NIC) continued to have significantly lower body weight which suggests the addition of prenatal nicotine exposure may have exacerbated this effect seen in previous preclinical studies investigating individual exposure. This suggests that combined prenatal exposure during early pregnancy may significantly impair offspring body weight and growth throughout adolescence.

Prenatal exposure of alcohol and nicotine during early pregnancy additionally altered developmental milestones, including ear unfurling, teeth eruption, and eye opening. Offspring with any prenatal alcohol exposure (EtOH+NIC, EtOH+VEH) had significantly delayed ear unfurling and eye opening. Our finding in eye opening is consistent with similar literature examining prenatal alcohol exposure alone (Thomas et al., 2009); however, Thomas et al. indicated no effects in ear unfurling which is not consistent with the current finding. Additionally, offspring with combined prenatal exposure (EtOH+NIC) experienced significant delays in teeth eruption, which is inconsistent with previous research. The same article by Thomas et al. (2009) did indicate prenatal alcohol exposure delaying teeth eruption; however, other research examining nicotine exposure during early childhood indicated early teeth eruption (Hanioka et al., 2018) which contradicts the current finding.

However, developmental milestone outcomes may be dependent upon methods such as dosage of drug, timing of drug exposure, routes of administration, and polydrug exposure. Importantly, there is sparse research on combined prenatal exposure during the first trimester. Prenatal alcohol exposure is associated with delayed teeth eruption; therefore, the addition of prenatal nicotine exposure could have further exacerbated this effect, ultimately delaying teeth eruption within offspring. Taking all milestone results together, prenatal alcohol exposure during the first trimester delayed ear unfurling and eye opening, with the interactive effects of combined prenatal exposure delaying teeth eruption.

Drug-Seeking Behavior Paradigm

Offspring body weights, food intake, and water intake were measured 2 days prior to beginning drug-seeking behavior testing and throughout the paradigm. Results examining body

weight indicated that overall, subjects with combined prenatal exposure (EtOH+NIC) generally weighed significantly less than all other prenatal exposure groups. This effect was similar to the body weight differences observed during earlier time points. In clinical literature, some people with individual exposure to alcohol and nicotine with low birth weight have been shown to eventually overcome this with age (O’Keeffe et al., 2015, Reeves et al., 2008, White et al., 2022). However, the opposite has also been shown. Prenatal nicotine exposure has been linked to obesity later in life that persists into adulthood (White et al., 2022). In contrast, prenatal alcohol exposure is associated with early growth restrictions in body weight that can also persist into adulthood (Carter et al., 2013); however, Carter et al reported this finding as being dose-dependent, where this was only seen within heavy maternal alcohol consumption during pregnancy. Due to our inability to analyze dams’ BAC’s at this time, it is unknown whether this is consistent with the current study.

Despite the inconsistencies with previous research to our findings, these studies investigated individual exposure to alcohol and nicotine and not combined exposure, which is sparse. Additionally, these previous findings contradict one another where prenatal alcohol exposure is associated with growth restrictions while prenatal nicotine exposure is associated with obesity. Due to these contradictions, we can only infer that the addition of prenatal nicotine exposure may further exacerbate growth restrictions caused by prenatal alcohol exposure, causing more severe growth restrictions within individuals with combined prenatal exposure of alcohol and nicotine.

During the self-administration paradigm, males overall had a significantly higher food intake compared to females, with no differences between subjects who were currently self-administering nicotine versus the e-cigarette vehicle. However, female subjects with any type of

prenatal nicotine exposure (EtOH+NIC, Air+NIC) ate significantly more compared to females with no prenatal nicotine exposure (EtOH+VEH, Air+VEH), again not differing based on what drug was being self-administered (SAM NIC, SAM VEH). These findings are consistent with previous research in clinical models (Werts et al., 2014, White et al., 2022), where separate prenatal exposure to alcohol or prenatal exposure to nicotine can increase food intake during adolescence. However, sex differences in previous research were only observed within offspring prenatally exposed to alcohol alone, with females being more likely to engage in constant snacking and more likely to meet criteria for obesity. These clinical data are relevant to our findings, since any prenatal nicotine exposure increased food intake, which includes co-exposure of both alcohol and nicotine.

Also during the self-administration paradigm, subjects with any type of prenatal alcohol exposure (EtOH+NIC, EtOH+VEH) generally drank less water compared to subjects with no prenatal alcohol exposure (Air+NIC, Air+VEH); this did not differ based on the self-administration drug (SAM NIC, SAM VEH). Significant differences were observed sporadically across days; however, no distinct pattern across days were detected. Furthermore, there is little to no research on the influences of prenatal alcohol exposure on offspring water intake leading us to infer that any prenatal alcohol exposure may decrease offspring water intake later in life.

During the self-administration paradigm for vaporized nicotine or the e-cigarette vehicle, subject's total pokes, valid pokes, and total vapes were recorded for each subject during self-administration. Total pokes consist of the total number of times the subject used to nose poke to try and self-administer their assigned drug. In contrast, valid pokes consist of the nose pokes that successfully activated vapor administration with the 10-second required timeout between vapes, which is the same as the total vapes during each session.

Overall, subjects that self-administered the e-cigarette vehicle (SAM VEH) tried to trigger the vapor via nose poke significantly more than subjects that self-administered nicotine (SAM NIC). This suggests that subjects sought out vehicle more compared to subjects receiving nicotine vapor, which is not surprising given that it may be perceived as sweet-tasting and fun to experience without the potentially aversive effects of added nicotine. It is notoriously challenging to train rodents to self-administer nicotine, as it can be perceived as aversive to them (Collins et al., 2012).

Overall, female subjects interacted with the nose poke hole more frequently compared to their male counterparts, with this effect especially prevalent among subjects that were self-administering nicotine. This suggests that females in this study may have displayed more severe drug-seeking behaviors compared to males, regardless of their prenatal drug exposure. Similar results were seen in the total number of vapes delivered, further supporting this interpretation. Previous research is consistent with some of our findings in this domain. For example, Rydell et al. (2012) reported that female with prenatal exposure to nicotine alone consumed nicotine more frequently compared to their male counterparts in clinical models.

Given that we did not observe any differences in drug-seeking behaviors among the prenatal drug exposure groups, these findings are not consistent with previous research. Previous literature has reported that individual prenatal exposure to either alcohol or nicotine increases drug-seeking behaviors within preclinical and clinical studies (Glantz et al., 2006; Malanga et al., 2003; Dodge et al., 2022), which is how we developed our original hypothesis. The lack of differences between prenatal exposure groups could have potentially been caused by some limitations of the SAM paradigm, such as no formal training being executed. Subjects were naturally curious and quickly self-administered their assigned drug which negated the need for

training and is not typical vapor self-administration training (Weimar et al., 2023). However, it is once again important to note that teratology research often varies in prenatal drug exposure type(s), dosage, timing, routes of administration, and behavioral outcome paradigms; thus, our results can only conclude that moderate prenatal exposure to alcohol and nicotine via e-cigarettes may not alter nicotine-seeking behaviors during adolescence. It should not be generalized across teratology and substance use disorder research as a whole.

Memory Performance

Analyses for memory performance using the novel object recognition (NOR) paradigm indicated that adolescent offspring exposed to prenatal alcohol alone (EtOH+VEH) in utero exhibited impaired memory performance by showing significantly more interest in the old object verses the novelty object. Furthermore, subjects with prenatal nicotine exposure alone (Air+NIC) behaved similarly to EtOH+VEH subjects during NOR, although this finding was not statistically significant. These findings suggest that subjects exposed to alcohol alone in utero may experience significant memory impairments compared to combined prenatal exposure and nicotine exposure alone during early adolescence. However, they were significantly more active compared to all other exposure groups which may contribute to this significant difference.

These findings are consistent with previous research examining individual prenatal drug exposure to alcohol or nicotine. Within clinical studies, reduced hippocampal volume have been observed with individuals with prenatal alcohol exposure alone, which is associated with learning and memory (Glantz et al., 2006; Solar et al., 2022). The literature has also shown similar findings with individuals with prenatal nicotine exposure alone experiencing poor overall cognitive performance (Levin et al., 1996). Given this previous research, we had hypothesized

that combined prenatal exposure would further exacerbate memory impairments, given that individual drug exposure exacerbates memory impairments. However, this hypothesis was not supported in the current study.

Results from the NOR paradigm during adulthood yielded slightly different results from those observed when offspring were adolescents. Only adult subjects with prenatal nicotine exposure alone (Air+NIC) had a significantly smaller discriminatory index compared to the control condition only (Air+VEH). However, follow-up analyses showed that adult subjects with any nicotine exposure (EtOH+NIC, Air+NIC) were also more active overall and interacted with each object (old, novel) more. The impaired memory performance in the adult Air+NIC subjects could replicate previous findings where prenatal nicotine exposure is associated with poor overall cognitive performance (Levin et al., 1996), or the impaired performance could be associated with increased activity levels.

Importantly though, none of the subjects in this study exhibited significant learning during the task, as there were no significant differences in old vs novel object interaction times in adolescence or adulthood. This could be due to several limitations during the task, including subjects not having an acclimation period of the box itself prior to experimental testing, and overall potential object preference. Despite no subjects exhibiting significant learning, adolescent subjects with prenatal alcohol exposure alone did interact significantly more with the familiar object compared to the novelty object, exhibiting memory impairments. Research examining prenatal alcohol exposure on memory performance have reported reduced hippocampal volume within subjects (Glantz et al., 2006; Solar et al., 2022) which is responsible for memory and may have caused this effect. More memory impairments were observed amongst adolescents, which

may have been caused by synaptic pruning and the continuation of brain development (Juárez-Portilla et al., 2018).

Less impairments were observed within adult subjects, suggesting that these effects may have diminished throughout development. However, adult offspring with prenatal nicotine exposure alone did show more discrimination between the objects but follow-up results indicated no significant differences amongst familiar versus novelty object interaction time. Similar findings were observed within adolescent subjects with prenatal nicotine exposure alone but were only near significant. Taking this into consideration, the effects of prenatal alcohol alone exposure may diminish during development while the effects of prenatal nicotine exposure may linger through adulthood.

Importantly, there is little to no research on combined prenatal exposure on memory performance; therefore, we can infer that the combination may not exacerbate the effects of individual exposure like we hypothesized. Furthermore, research on prenatal nicotine exposure evaluates overall cognitive impairments including memory while research on prenatal alcohol exposure focused more on memory and hippocampal brain areas. Overall, the current findings reflect supporting research of individual prenatal exposure impairing memory performance, specifically within adolescent subjects.

Offspring Tissue

Offspring body, brain, liver, heart, and lung weights were recorded once behavioral tasks were completed for all subjects. Body weight analyses indicated that male offspring with combined prenatal exposure (EtOH+NIC) weighed significantly less compared to all other groups (EtOH+VEH, Air+NIC, Air+VEH). No significant differences in prenatal alcohol and/or

nicotine exposure were observed among female offspring. However, it is possible that the smaller range of body weights among females made it not possible to detect more subtle differences among the prenatal exposure groups, which could be observed among males which are larger overall and have a wider range. Regardless, the long-term reduced body weights among male offspring co-exposed to prenatal alcohol and nicotine is consistent with earlier findings from this study, which concluded that combined prenatal exposure to alcohol and nicotine may cause growth restrictions.

Offspring Brain Weight

Analyses examining offspring brain weight indicated that males with combined prenatal exposure (EtOH+NIC) had significantly lower brain gross weight compared to all other exposure groups, with no differences among female offspring. This is consistent with previous research, where individual prenatal exposure to alcohol or nicotine caused reduced brain gross weight (Bassi et al., 1984, Marroun et al., 2013). However, no sex differences were observed in either of these previous studies.

Offspring Liver Weight

Analyses for gross liver weight indicated that females with any nicotine exposure (EtOH+NIC, Air+NIC) had larger gross liver weights compared to alcohol exposure alone (EtOH+VEH). In contrast, males with combined prenatal exposure (EtOH+NIC) had significantly smaller liver weights compared to males with prenatal nicotine exposure alone (Air+NIC). These findings contradict previous research, as studies have reported that prenatal nicotine exposure causes reduced liver weights (Marroun et al., 2013, Bassi et al., 1984), while

studies on prenatal alcohol exposure reported no differences in gross liver weight compared to their control group (Huebner et al., 2016). However, it is important to note that gross weights are not the best indicator of volume differences, and the methods of these previous research studies vary greatly from the current study.

Offspring Heart Weight

No significant differences in prenatal alcohol or nicotine were observed in offspring heart weight; however, general sex differences were notable. Females had significantly smaller hearts compared to males, which is consistent with previous research. Previous research examining general size differences between female and male hearts reported males having significantly larger hearts compared to females, regardless of overall height and weight in the individual (Westaby et al., 2022). However, these findings are not consistent with previous research examining individual prenatal drug exposure of alcohol and nicotine. Previous research reported that, individually, alcohol and nicotine may decrease heart weight within offspring, regardless of sex (Bassi et al., 1984, Ninh et al., 2019).

Offspring Lung Weight

Analyses examining offspring lung weight indicated that females with any prenatal alcohol exposure (EtOH+NIC, EtOH+VEH) had significantly smaller lungs compared to females with prenatal nicotine exposure (Air+NIC), with no significant differences in prenatal alcohol or nicotine within male offspring. This is consistent with previous research, which reported that prenatal alcohol exposure causes smaller lung weights (Wang, et al., 2007); however, this study examined fetal lung weight only. Furthermore, research examining lung weight in postnatal

lambs reported no significant differences compared to the control group (Sozo et al., 2010). Research examining prenatal nicotine exposure on lung weight reported smaller lungs weight in offspring with prenatal nicotine exposure compared to the control group (Bassi et al., 1984), which may have contributed to the current findings of combined prenatal exposure. None of the literature discussed reported any sex differences in prenatal alcohol or nicotine exposure, which is not consistent with the current findings.

Limitations

There are several limitations to address in this study. First, the control condition (Air+VEH) still underwent the same environmental stressors as the other experimental groups (EtOH+NIC, EtOH+VEH, Air+NIC), which could affect data outcomes (Lightfoot et al., 2024). Future or follow-up studies should include an additional control condition where pregnant dams do not experience any vapor exposure or additional stressors (i.e. blood collections). Secondly, due to delayed shipment and Analox troubleshooting, dam BACs could not be analyzed for this thesis defense date, meaning that target BACs could not be confirmed for all dams.

Third, subjects within the self-administration paradigm were naturally curious and quickly self-administered their assigned drug. This is not typical in vapor self-administration paradigms (Weimar et al., 2023), and negated the need for proper training. Based on the visual graphs, some subjects self-administered multiple times on the first day of testing but quickly become bored and self-administer very little during the remaining days. Subjects not properly being trained may have contributed to the lack of data differences among prenatal exposure groups; therefore, any follow-up studies should take this into account and fully train subjects to use the nose poke regardless of baseline behaviors.

Fourth, there was no acclimation day for the plexiglass box used in the NOR paradigms prior to memory performance testing. The lack of discrimination between objects could have resulted from raw curiosity about their new surroundings. Fifth, the same objects for NOR were used throughout this study; therefore, it is unknown if subjects had a preference towards the old object compared to the novelty object. Further follow-up studies should add an acclimation day prior to testing and switch objects to avoid preferences. Lastly, coders inner rate reliability analysis was slightly below the acceptable range (90%+) which could have negatively impacted NOR data.

Strengths

Importantly, there are several strengths of this study design. Using a preclinical model allowed us to carefully control the environment and maintain consistency throughout the study, including administering alcohol and/or nicotine at consistent doses during pregnancies. Having a carefully controlled environment removes potential environmental confounds that are found within clinical models, such as homelife, peer pressure, stigma, self-report data, etc. We also ensured that all tasks were executed within the same time frame to eliminates time-dependent outcomes, and all behavioral tasks were executed by the same individual(s) to avoid potential handling stress within subjects.

Additionally, this study used a vapor inhalation model that is clinically relevant, using e-cigarette tanks purchased from local smoke shops and customizing the nicotine e-cigarette liquid to reflect dosages commonly consumed today. Moreover, using vapor inhalation during both the prenatal exposure paradigm and the adolescent self-administration paradigm minimized potential confounds of stress that are commonly observed from other routes of administration, such as

injections, drinking, and intragastric intubations. Most importantly, this is the first study to examine the effects of prenatal co-exposure to alcohol and nicotine via e-cigarettes on later-life drug-seeking behaviors and potential memory impairments in a rodent model; previous research has historically focused on the effects of one prenatal exposure at a time.

Conclusion

Overall, the current study suggests that prenatal drug exposure to alcohol and/or nicotine via e-cigarettes during the first trimester of pregnancy alters maternal body weight, food intake, core body temperature, offspring development, offspring food and water intake, drug-seeking behavior, memory performance, and gross organ weight in domain- and sex-specific manners. Alcohol exposure during early pregnancy decreased maternal body weight gain; however, nicotine exposure caused an increased food intake which may contribute to nicotine exposed dams having greater body weight gain. Dams exposed to nicotine had lower starting core body temperatures during the vapor inhalation paradigm, which may have contributed to their less drastic temperature changes. In contrast, combined prenatal exposure to alcohol and nicotine had significant effects on offspring body weight gain and body length throughout their development.

Combined prenatal exposure additionally delayed teeth eruption, whereas prenatal alcohol exposure alone delayed ear unfurling and eye opening. Later in life, neither prenatal exposure to alcohol or nicotine via e-cigarettes did not alter offspring drug-seeking behavior, although females generally exhibited more drug-seeking behaviors compared to males, and all subjects preferred the vehicle over nicotine vapor. Prenatal exposure to alcohol alone increased overall activity and object interactions during adolescence, and prenatal exposure to nicotine alone had similar effects during adolescence and adulthood; these activity level differences may have contributed to the impaired memory performance observed among these groups. Combined prenatal exposure did not alter memory performance. Taken together, this data suggests that prenatal drug exposure to alcohol and/or nicotine via e-cigarettes may have sex- and domain-specific effects during pregnancy and throughout offspring development that are individual, additive, and interactive.