EFFECTS OF DEVELOPMENTAL LOW-LEVEL LEAD EXPOSURE ON VOLUNTARY ALCOHOL CONSUMPTION AND DRUG-INDUCED BEHAVIORAL SENSITIZATION IN ADULTHOOD

by

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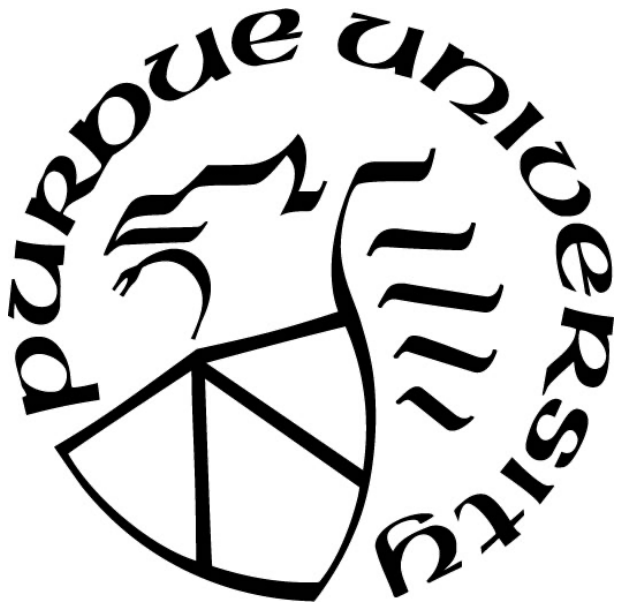

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Dedicated to my incredible family and friends. I love y'all.

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ABSTRACT

Lead (Pb) is one of the most harmful and most abundant neurotoxins in the environment. Despite the extensive movement made to eradicate toxic levels of Pb in the environment, children, predominately in lower socioeconomic areas, are still exposed to varying levels of Pb. Human studies suggest that Pb exposure leads to altered drug consumption in adults by altering underlying neural mechanisms, specifically dopamine (DA) activity. However, there is limited research on this at blood Pb levels below 10 μg/dL, levels often seen in children growing up in neighborhoods located in old industrial and urban areas. To model how early-life low-level Pb exposure effects DA-dependent behaviors associated with addiction in adulthood, we used C57BL/6J mice. Litters were weaned at PND 21 and assigned to either a three-week history of 30 parts per million (ppm) Lead (IV) Acetate exposure or a control condition of 0 ppm Pb in DI drinking water. After the Pb exposure period, mice were switched to regular tap water until they reached adulthood. Afterward, separate animals were tested in one of three experiments: two-bottle choice alcohol preference drinking, alcohol-induced behavioral sensitization (EBS), and cocaine-induced behavioral sensitization (CBS). In experiment 1, our hypothesis was met, and both male and female mice with a prior Pb exposure displayed significantly higher alcohol intake and preference scores over the three-week period than control mice. In experiment 2, there were no differences in EBS and no evidence of EBS in any of the groups. However, there was an increased acute response to 2.0 g/kg EtOH in the Pb-exposed chronic group as compared to the control animals. Lastly, in experiment 3, Pb-exposed animals in the chronic cocaine group were more sensitive to the effects of cocaine (10 mg/kg) across days than the controls, both the acute cocaine groups and both saline control groups. Thus, with these experiments, we concluded that low levels of developmental Pb exposure might be targeting DA in the reward pathway, which is essential for alcohol intake and drug sensitization.

INTRODUCTION

Lead Exposure Overview

Lead (Pb) is one of the most harmful heavy metals ubiquitously found in the environment. There is a large body of evidence suggesting that Pb persists as a significant health concern in the United States (Dignam et al., 2019; Filippelli et al., 2015). Despite extensive progress made to eradicate toxic levels of Pb in the environment, populations living in lower socioeconomic areas are exposed to varying levels of Pb at much higher rates than other populations (Sanders et al., 2009; Sobin et al., 2013). Increased exposure to Pb often occurs through drinking water, soil, air pollution, old pipes, and paint (Lanphear & Roghmann, 1997; Virgolini & Cancela, 2014).

Lead enters the bloodstream primarily via ingestion, with secondary pathways including inhalation and absorption through the skin. Initially, Pb bonds to hemoglobin and travels in the blood, eventually making its way to soft tissues, such as the brain and liver. While most of the Pb in the blood of an adult can clear from the system through waste in a short amount of time, only about 32% of the lead in a child will leave the system this way (Barry, 1975). Moreover, Pb is stored and accumulates in hard tissues, such as bones and teeth, for extended periods (Sanders et al., 2009). Lead can accumulate in bone over time, which turns into a storage site for the heavy metal and makes it difficult to eliminate from the body. As a result, Pb can reenter the bloodstream and travel to other areas disrupting normal functions, such as neuronal firing. The half-life of Pb in bone, that is, the clearance of Pb from the body, has been reported to range from 10 to 30 years in adult humans (Rabinowitz, 1991). This differs in children because of the continuous growth or development of the bones. During this remodeling, Pb is continuously released back into the bloodstream. Specifically, it has been found that Pb stored in bone can account for as much as 90% of the Pb seen in the blood (Gwiazda et al., 2005). Thus, assessing the half-life of Pb in children is difficult because bone levels and blood levels of Pb vary over time as a result of accumulation (Barbosa et al., 2005; Barry, 1975).

Toxic blood lead levels (BLL), now identified as BLLs greater than 10 μg/dL (Center for Disease Control and Prevention, 2015), target the central nervous system (CNS) and impair neurodevelopment, cognition, motor functions, and several neurobiological mechanisms (D. A. Cory-Slechta, 1995; Jones & Miller, 2008; Mason et al., 2014; Shukla et al., 1989). Moreover,

there is no known safe threshold of Pb (Mattalloni et al., 2019; Virgolini et al., 2019). A growing body of epidemiological and experimental studies have consistently found that the presence of low levels of Pb (<10 μg/dL) during development may alter behavioral and neurobiological systems that are long-lasting and persist into adulthood (Jones & Miller, 2008; Nation et al., 2003). This information suggests that a developing organism is more vulnerable (Moreira et al., 2001). Therefore, more careful considerations of BLLs in children have been implemented, and the CDC now recommends health services and monitoring be encouraged for children with a BLL ≥5 μg/dL (CDC, 2017).

Currently, the average BLL of children living in lower socioeconomic environments throughout the United States is between 2.5 and 7 μg/dL (Nigg et al., 2010; Sobin et al., 2009, 2013). Several human studies have shown cognitive deficits and neurological changes in children with BLLs as low as 2 μg/dL (Canfield et al., 2003; Needleman & Bellinger, 1991; Sobin et al., 2013). However, it is difficult to study the effects of lower levels of Pb in children and the effects at varying life stages as behaviors may not manifest immediately; this results in fewer children undergoing testing (Canfield et al., 2003; Needleman & Bellinger, 1991). Further, there are also limitations due to small sample sizes, timing, and cost (Needleman & Bellinger, 1991). Thus, there is a need to further understand if or how BLLs below the current recommended threshold also lead to long-lasting behavioral changes using alternative models, such as animal models.

Lead Exposure and Substance Use Disorders (SUD)

Underserved and/or economically disadvantaged populations living in industrial environments, which contain higher concentrations of Pb via soil and air pollution or, otherwise, are more vulnerable to substance use disorders (SUD) due to various mediating factors (Enoch, 2006; Kadushin et al., 1998; Karriker-Jaffe, 2011). The development of SUDs is highly complex and often characterized by a combination of genetic and environmental factors (Prom-Wormley et al., 2017). Environmental factors mediating the development of SUDs include stress, living conditions, peer pressure, adverse life experiences, parental attitudes, and the age of a person's first exposure (Enoch, 2006). These factors interact with genetic factors, especially during adolescence, increasing an individual's initial use of a substance and eventual development of a SUD. While limited, a handful of studies have provided evidence that toxic levels of early-life chronic Pb exposure are positively correlated with juvenile delinquency, increased substance use,

and increased anxiety (Dietrich et al., 2001; Fishbein et al., 2008; Needleman & Bellinger, 1991; Pegues et al., 1993). An early study, using data from a population in Switzerland, found that participants who consumed alcohol more frequently (daily) had higher average BLLs than participants who were abstinent fish (Probst-Hensch et al., 1993). Other studies have found similar correlations (Pizent et al., 2001); however, the mechanisms by which Pb alters the brain and behavior at varying levels over time are not fully understood.

Preclinical models have consistently shown that early life Pb exposure is associated with an increased proclivity to drug addiction. Specifically, Pb exposure yielding BLLs >10 μg/dL have shown increased sensitivity to cocaine, increased lever pressing for heroin and morphine, and increased voluntary consumption of ethanol (EtOH) (Mattalloni et al., 2017; Nation et al., 2000; Virgolini et al., 1999). Additionally, several preclinical studies have found that endophenotypes of substance use disorders, such as anxiety, depression, risky behavior, and impulsivity, are highly correlated with varying levels of Pb exposure (Rocha et al., 2004; Virgolini & Cancela, 2014). While there is evidence that high BLLs are correlated with altered drug response in preclinical models, a gap in the literature remains about how or if low-level Pb exposure leads to these changes.

Lead Effects on Dopamine

Lead crosses the blood-brain barrier (BBB) by mimicking Ca^{2+} ions (Florea et al., 2013; NourEddine et al., 2005). There is no known mechanism by which Pb is transported across capillary endothelial cell membranes (Cheong et al., 2004; Kerper & Hinkle, 1997); however, there are early studies suggesting that the Ca^{2+}-ATPase pump plays an important role in this mechanism (Bradbury & Deane, 1993; Kerper & Hinkle, 1997). Once in the brain, Pb disrupts Ca^{2+} homeostasis, alters normal neurotransmission pre- and post-synaptically, and gene transcription (Florea et al., 2013; Jones & Miller, 2008; Zuch et al., 1998). Specifically, because Pb competes with and substitutes Ca^{2+}, there can be an accumulation of Ca^{2+} in cells that have been exposed to Pb, which can initiate apoptosis (Florea et al., 2013; Lidsky & Schneider, 2003). Moreover, presynaptically, Pb affects Ca^{2+} channels important for transmitter release (Lidsky & Schneider, 2003; Nachshen, 1984). Briefly, without Ca^{2+}disruption by Pb, action potentials cause Ca^{2+}channels to open, increasing Ca^{2+} in a presynaptic cell and, this along with the involvement of proteins such as calmodulin, activate synaptotagmin to drive the fusion of the synaptic

vesicles with the plasma membrane, which then releases the neurotransmitters (Florea et al., 2013). Pb prevents or disrupts this fusion and, as a result, inhibits neurotransmission. Disruptions in neurotransmission within the mesolimbic dopamine pathway, known as the reward pathway, may address why Pb produces an altered response to drugs of abuse and behaviors associated with addiction (Nation et al., 2000; Virgolini et al., 2017; Virgolini & Cancela, 2014).

Despite the differences in neurochemical targets and in behavioral responses, all drugs of abuse affect dopamine (DA) neurons in various brain structures. Specifically, DA is known to play an important role in reward and motivation in several structures associated with addiction. Within the reward pathway, DA neurons from the VTA project to and terminate at the nucleus accumbens (NAc), while also projecting to other target neural substrates such as the bed nucleus of stria terminalis (BNST), amygdala, lateral septal area, and lateral hypothalamus (Adinoff, 2004; Broadbent, Kampmueller, & Koonse, 2005; Phillips, Pastor, Scibelli, Reed, & Tarragon, 2011). Studies have shown that chronic EtOH and sensitization to various drugs of abuse increase the amount of DA in the NAc, which could be mediated through secondary messengers or other proteins. Moreover, several studies have shown that the mesolimbic DA pathway contributes to neuroadaptive changes as a result of drug-induced behavioral sensitization; however, the specific role that D1 and D2 receptors have on the acquisition and expression phases of drug-induced behavioral sensitization (DBS), separately, are less clear.

Lead-induced changes in the DA system are a result of enhanced or attenuated DA neurotransmission (Jones & Miller, 2008; Mattalloni et al., 2017; Nation et al., 2000), which is why developmental exposure is hypothesized to be linked to substance use disorders. These changes in DA neurotransmission are, in part, due to increased DA turnover and receptor super-sensitivity (Jones & Miller, 2008; Nation et al., 2000, 2003; Stansfield et al., 2015). For example, Pb exposure during gestation and lactation lead adult animals to self-administer cocaine at higher rates than non-exposed animals, which is evidence of Pb enhancing behaviors such as drug-seeking and taking (Nation et al., 2004; Robinson & Berridge, 2008). Moreover, Nation et al. (2000) have shown that early Pb exposure at high levels both decreases and increases an animal's locomotor-stimulant response to cocaine at different ages, which relies heavily on DA as cocaine blocks the reuptake of this neurotransmitter, increasing DA activity. However, the exact mechanisms underlying these opposing results are not yet known.

Additionally, in vivo and in vitro studies that focused on the reward pathway have found that Pb exposure disrupts dopamine neurotransmission across different stages and is dependent on the amount and duration of Pb exposure (Jones & Miller, 2008; Toscano & Guilarte, 2005; White et al., 2007). For example, Gedeon et al. (2001) conducted a study in rats exposed to Pb for either 0, 30, 60, 90, 120, and 180 days. After exposure, they found that after 30, 60 and 90 days, there was a hypodopaminergic state or decrease in D2 receptor expression in the NAc, but after 180 days, there was an increase in D2 receptor expression. These findings are in line with other studies, such as Pokora et al. (2002), which found that Pb exposure decreased D1, D2, and dopamine transporter (DAT) binding sites in the NAc. However, there are discrepancies between these studies as reductions in D2 and DAT binding sites were seen after two-weeks of Pb exposure and remained that way until the 12-month period, while D1 reductions were evident only after eight months of exposure and were resolved by the 12-month period (Pokora et al., 2002). Further, consistent with Gedeon et al. (2002), a study that used D1 and D2 receptor antagonists to target how Pb disrupted DA binding, results showed a significant decrease in D2 activity, indicating that D2 receptors appear to be more sensitive to Pb than D1 receptors (Ma et al., 1999). These data suggest that Pb targets receptors differently; however, it is important to note that discrepancies and inconsistencies across studies could be due to differences in methodology.

Regardless, these data are compelling and confirm that DA activity is altered with Pb exposure; however, no studies looking at behaviors associated with addiction have used animal models yielding BLLs lower than 10 μg/dL. Given that Pb exposure targets and disrupts DA neurotransmission, studying different DA-dependent behaviors linked to addiction could add to the current, limited body of literature focusing on SUDs and contribute information that could lead to a further understanding of how these underlying mechanisms change over time as a result of Pb-induced neural disruptions during development.

Lead Exposure, Ethanol Exposure, and Anxiety-like Behaviors

Alcohol remains one of the most abused substances in the world (World Health Organization, 2018), and preclinical models have found that Pb exposure (yielding BLL >12 μg/dL) during development increases the amount of alcohol voluntarily consumed using various paradigms (Nation et al., 1986; Virgolini et al., 2017, 2019; Virgolini & Cancela, 2014). More

recently, Mattaloni et al., 2019 found that perinatal Pb exposure, yielding BLLs around 6.5 μg/dL, the lowest in the current literature, increased ethanol consumption in periadolescent rats (postnatal day 35). One reason for this increase in consumption could be due to the disruption of DA neurotransmission by Pb.

Studies have shown that chronic EtOH consumption and drug-induced behavioral sensitization (DBS) increases the amount of DA in the NAc (Nashed et al., 2019; Quadros et al., 2002; Souza-Formigoni et al., 1999). Additionally, electrophysiological experiments have shown that acute EtOH enhances the firing rate of VTA DA neurons at different doses (Didone et al., 2016). This enhancement of DA firing can be attributed to EtOH specifically because this activity will also occur when inputs from surrounding neurons are blocked (Brodie et al., 1990; Nimitvilai et al., 2012). Furthermore, animals have also been shown to lever press for 10% ethanol at higher rates when exposed to Pb than those that are not (Virgolini & Cancela, 2014). This evidence demonstrates that Pb potentially changes seeking behavior as well.

An alternative explanation for an increase in EtOH consumption could be due to the anxiolytic properties produced by EtOH. Anxiety is an endophenotype of addiction and has been widely studied in conjunction with EtOH consumption (Kliethermes, 2005). Anxiety has shown to positively correlate with bone Pb levels in humans (Rhodes, Spiro, Aro, & Hu, 2003), and increased anxiety has also been found in Pb-exposed animals (Ferlemi et al., 2014; Shvachiy et al., 2018). In the preclinical literature, however, there have been conflicting findings such that two studies did not find an increase in anxiety-related behavior in animals with Pb exposure (Moreira et al., 2001; Virgolini et al., 1999).

In an unpublished study from our lab, we found that developmental Pb exposure increases anxiety-like behaviors in adult mice via the EPM test at PND65+ (unpublished, 2018). Moreover, Shvachily et al. (2018) found that there was a significant decrease in the number of open arm entries in the elevated plus maze (EPM) made by the Pb-exposed group compared to the control group, indicating greater anxiety-like behavior in rats. Further, other preclinical studies have found that there are sex differences among mice tested using the open-field test (OFT) after a period of Pb exposure (Kasten-Jolly et al., 2012). Kasten-Jolly et al. (2012) found that females were more anxious, based on a decrease in the time spent and time traveled in the center, compared to males. Thus, one possibility could be that the anxiety-like behaviors induced by early Pb exposure promote EtOH intake in adult mice.

While these studies provide compelling evidence that Pb exposure is altering drug response at levels previously overlooked, more research is needed in order to investigate translational levels seen in children today. Further, of the studies that have looked at EtOH consumption, the Pb exposure period was during gestation and through lactation. In the scope of childhood Pb exposure, this type of model is a confound because there is evidence that the effects of Pb exposure may manifest differently when exposed gestationally compared perinatally (Zenick et al., 1979). Thus, different models looking at perinatal Pb exposure are needed to explore the effect of childhood Pb exposure later on in life.

The Effects of Lead Exposure on Drug-induced Locomotor Sensitization

Repeated administration of drugs at a fixed dose increases the behavioral effects induced by drugs over time. This persistent and long-lasting neuroadaptive increase in behavioral response is attributed to the phenomenon deemed behavioral sensitization, which is a DA-dependent behavior as well (Berridge, 2007; Camarini & Pautassi, 2016; Robinson & Berridge, 1993). Historically, this was thought of as a model of drug-induced neuroplasticity because of the gradual changes (Lüscher & Malenka, 2011; Phillips et al., 2011). Drugs such as cocaine, methamphetamine, and morphine have consistently shown to increase the stimulatory effects of the drug in preclinical models.

In rodents, behavioral sensitization is modeled by measuring locomotor activity over a selected amount of time, while in humans, self-reports of sensitized vigor and energy are measured (Phillips et al., 2011). Ethanol-induced behavioral sensitization (EBS) has been linked to the pivotal transition from regular alcohol consumption to an alcohol use disorder diagnosis via increased incentive salience or an increase in drug-seeking/wanting behavior (Camarini & Pautassi, 2016). Once these cellular neuroadaptive patterns arise, individuals vulnerable or at risk for alcohol use disorder (AUD) are then put into a motivational state, which in turn increases the likelihood of compulsive behavior and/or relapse in patients with AUD (Camarini & Pautassi, 2016; Didone et al., 2019). One limitation to EBS is that sensitization is dependent on the genotype and protocol used. Given that Pb exposure may mediate EtOH consumption through alterations in DA activity, EBS may be affected as well. Currently, there are no articles focusing on the effects of Pb exposure on EBS specifically; however, DA supersensitivity as a result of Pb exposure may result in an increased sensitivity to EtOH in this behavioral paradigm.

Lastly, cocaine-induced behavioral sensitization (CBS) is also DA-dependent, and repeated cocaine exposure more consistently produces behavioral sensitization in mouse models as compared to EtOH. Similar to EBS, CBS also produces changes in the mesocorticolimbic system and potentiates motivation or promotes a "wanting" for drugs (Robinson & Berridge, 2008). While EBS does involve DA activity, EtOH's primary target is not DA. Alternatively, DA is the primary target of cocaine, along with serotonin and norepinephrine (Koob, Arends, & LeMoal, 2014).

Cocaine has a specific mechanism of action that is well established in the literature, and there is more information about the role of DA in CBS models as well. Thus, this behavioral test could be useful in elucidating whether translational levels of Pb in children alters DA. Cocaine mimics DA and acts as a competitive DA agonist, binding to the dopamine transporter (DAT), which results in an increase of DA in the synapse and a decrease in DA reuptake (Koob et al., 2014).

There have been few studies to look at the effects of Pb exposure on cocaine sensitivity and sensitization. One study found that prenatal through perinatal Pb exposure yielding BLLs greater than 15 μg/dL was shown to increase cocaine sensitivity at PND 30 in periadolescent rats (Nation et al., 2000). However, within the Pb literature, one caveat is that the methodologies are variable and produce varying BLLs across studies, which makes it difficult to compare and interpret findings. Currently, no studies are focused on the effects of lower-level Pb exposure on cocaine sensitivity or CBS. Thus, to further assess if Pb exposure at these translational levels is acting on DA activity, elucidating how it will affect CBS is promising.

Rationale

While there is a growing body of evidence that low levels of developmental Pb exposure alter behaviors that are endophenotypes of addiction, several gaps remain in the literature. Our interest lies in the question of whether chronic, low-level Pb exposure (ending during periadolescence) would result in underlying mechanistic changes that would then alter behavioral responses to drugs in adulthood (PND 65+). In the current body of literature, there is evidence that Pb exposure alters DA-dependent behaviors; however, differences in methodological procedures, as well as the translational aspect of BLLs, are some of the limitations. Few studies have exposed animals to Pb perinatally and have instead exposed them

through gestation or lactation, which asks a different question than the present study. Further, some of the studies of these behavioral findings have used stressful methodologies to treat animals with Pb, such as oral gavage.

Additionally, it is difficult to draw conclusions from these experiments as most Pb exposure periods varied in terms of time and duration, which results in contrasting findings or differences across similar behavioral tasks (Nation et al., 1986; Virgolini & Cancela, 2014; Zenick et al., 1979). Sobin et al. (2013) developed a three-week model of maternal Pb exposure through drinking water that has consistently yielded BLLs < 6 μg/dL in pups, which are BLLs seen in children living in industrial cities like Indianapolis, IN (Filippelli et al., 2015; Sobin et al., 2013). Thus, using this translational preclinical model of chronic low-level Pb exposure during a developmental period (PND 21-42), a series of experiments were conducted to explore whether this perinatal model of developmental Pb exposure results in similar increases in EtOH consumption and explore if Pb influences drug-induced behavioral sensitization, which is heavily dependent on changes in DA receptor activity.

Collectively, these experiments will add new information about the effects of lower BLLs in early life to behaviors associated with addiction in adulthood. Lastly, studying how low levels of Pb exposure alter drug responses later in adulthood in various behavioral models of addiction will allow us to investigate if the DA system is being altered at these lower levels and conclude if they result in longitudinal behavioral changes.

Specific Hypotheses

The purpose of aims 1-3 is to explore the effects of low-level developmental Pb exposure on DA-dependent behaviors in adulthood. We hypothesize that low-level Pb exposure will result in an increase in voluntary EtOH consumption and preference for EtOH in a two-bottle choice (2BC) paradigm. Also, given that drinking may be driven by anxiety-like behaviors, we hypothesized that Pb exposed mice will exhibit greater anxiety-like behaviors as measured by the elevated plus maze (EPM) than controls and that a history of EtOH would potentiate these behaviors due to a 24-hr abstinence. In Aims 2 and 3, we wanted to investigate the effects of early-life Pb exposure on drug-induced behavioral sensitization. Aim 2 focuses on EBS, where we hypothesized that Pb exposure would lead to increased sensitivity to EtOH and have a more robust sensitization curve than controls. Further, we also hypothesized that differences in

anxiety-like behaviors seen in EPM pilot studies would replicate using an open field test (OFT). In Aim 3, we hypothesized that Pb exposure would increase sensitivity to cocaine across days and that after one week of withdrawal to cocaine, Pb exposed mice would exhibit a continued heightened response to 10 mg/kg cocaine than control animals.

MATERIALS AND METHODS

Subjects

All animal procedures were approved by the Indiana University-Purdue University Indianapolis (IUPUI) Institutional Animal Care and Use Committee (IACUC). Postnatal day (PND) 21 C57BL/6 mice from our internal breeding colony (IUPUI) were weaned and assigned to receive either 30ppm or 0ppm of lead (IV) acetate (Sigma Aldrich, St. Louis, MO, USA) in distilled water. This protocol was adapted from Sobin et al. (2013) and Flores-Montoya et al. (2015), where such pre- and postnatal exposure resulted in BLL ranging between 2–5 μg/dL in the 30ppm group. Males and females were counterbalanced by litter to account for litter effects. Mice were group-housed in standard mouse cages with wood chip bedding (Sani-Chips; PJ Murphy Forrest Products Corp., Montville, NJ) under a 12-hour reverse light/dark schedule in temperature- and humidity-controlled rooms. Mice assigned to the experimental group also had a standard filter top throughout the three-week exposure period and two weeks after. Treatment to 30 ppm or 0ppm exposure lasted three-weeks (until PND 42) at which the control fluid was switched to standard glass water bottles filled with tap water, and animals were left undisturbed until they reached at least PND 62. All mice had ad libitum access to food and water for the duration of the experiments. A breakdown of the number of animals in each experiment can be seen in Table 1.

In Experiment 1, a total of 56 C57BL/6 mice (28 males and 28 females) were tested, and, of these, half were exposed to 30 ppm Pb. Mice were single-housed once they were at least PND 62 and randomly assigned to a 3-week EtOH history or 0-week history group. Mice were PND 66 –99 at the start of experimentation.

In Experiment 2, a total of 96 C57BL/6 mice (57 males and 39 females) were tested. Animals remained pair- or trio-housed throughout the experiment and were counterbalanced by sex, age, and litter in each experimental group. Mice were PND 63 – 86 at the start of experimentation.

In Experiment 3, a total of 61 C57BL/6 mice (31 male and 30 female) were tested. Animals were single-housed and randomly assigned to one of three experimental groups. Mice were PND 95 –108 at the start of experimentation.

Drugs and Drinking Solutions

The ethanol solution used in experiment 1a was made by diluting 200 proof ethanol (Pharmco Inc., Brookfield, CT) in tap water. For experiment 2, 190 proof ethanol (Pharmco Inc., Brookfield, CT) was diluted in 0.9% physiological saline. For experiment 3, cocaine hydrochloride was purchased from Sigma Aldrich (St. Louis, MO) and also diluted in 0.9% physiological saline.

Experiment 1

Two-bottle Choice (2BC) EtOH Drinking Procedure

In *experiment 1a*, animals were counterbalanced by sex, exposure condition, and litter, and assigned to either a three-week EtOH group (3W) or a control group (0W). In the 3W group, mice had 24-hour access to one bottle of 10% EtOH (in a 50 mL graduated cylinder) and one bottle of tap water (25 mL graduated cylinder) for 21 days. Animals in the 0W group had one 50 mL graduated cylinder with tap water, and one 25 mL graduated cylinder with tap water. Intakes were measured without disturbing the bottles every Monday, Wednesday, and Friday in order to determine the amount of ethanol and water consumed. On these days, the sides of the bottles were switched to deter animals from forming a side preference. One cage change occurred two weeks from the start of the experiment. Lastly, all mice were weighed at the beginning of the 2BC drinking phase and each Monday thereafter.

Apparatus

In experiment 1b, all animals were tested in an elevated plus maze (EPM) apparatus (Med Associates, St Albans, VT) consisting of two open (up to 76 cm long) and two opposite closed arms (up to 76 cm long with walls up to 20 cm high) elevated to 75 cm above the floor.

Animals were placed in the center of the apparatus and the number of open and closed arm entries, the amount of time spent in the open and closed arms, and the number of protected and unprotected head dips were measured for 5 minutes. Unprotected and protected head dips,

defined as a mouse looking down from an open arm or a mouse looking down, but remaining out of an open arm were scored blindly. Sessions were recorded using a camera mounted directly above the maze under red light that started at 1000, when the animal's usual lights off period ensued. The arms were cleaned with a Clidox solution between tests.

Experiment 2

EtOH-induced Locomotor Sensitization

The objective of experiment 2 was to assess the effects of developmental Pb exposure on EtOH-induced locomotor sensitization. Table 2 summarizes the injection days, groups, and days animals were placed in the locomotor boxes. Mice in experiment 2 underwent a 14-day behavioral sensitization protocol, which included the context paired with every injection. Day 0 was a 20-minute baseline locomotor and anxiety assessment with no injection and was analyzed separately from the sensitization protocol. Days 1-14 consisted of two habituation days (all saline injections; days 1 and 2), an acute day of 2.0 g/kg of EtOH or the saline equivalent (day 3), ten sessions of 2.5 g/kg EtOH or the saline equivalent (days 4-13), and a challenge day of 2.0 g/kg of EtOH or the saline equivalent (day 14). Each day, mice were moved to the behavioral testing room 4-hours into their dark cycle and were left undisturbed for 1-hour prior to the start of testing. All locomotor testing occurred under red light. During habituation, mice were injected with 0.9% physiological saline (i.p.) beginning at 1500 and were immediately placed into the Accuscan VersaMax activity monitors for 15 minutes. The baseline locomotor activity for days 1 and 2 was used to counterbalance mice for designation to one of three conditions for the remainder of the experiment: chronic ethanol (CE), chronic saline (CE), and saline control (SC). Injections were given daily since this has shown to be the most reliable way to achieve ethanol-induced locomotor sensitization in C57BL/6 mice (Phillips et al., 2011). The CE group received ten injections of 2.5 g/kg of EtOH on days 4-13, while the CS and SC groups received saline injections. On the challenge day (day 14), CE animals received a challenge injection of 2.0 g/kg of EtOH, the CS animals received their first injection of 2.0 g/kg of EtOH, and the SC animals were injected with saline. The saline control groups served as the experimental control for repeated injection stress on locomotor activity. In this case, sensitization can be characterized by looking at the difference between day 3 and day 14, which shows the classic definition of an

increase in locomotor activity over time after repeated injections of a drug. The CS group further allows for the assessment that repeated drug exposure is required in order to attain a sensitized response to a drug, which is used across different models of sensitization.

Activity Monitoring

For *experiments 2 and 3*, the locomotor activity of mice was monitored by 8 AccuScan VersaMax activity monitors (Omnitech Electronics Inc., Columbus, OH) controlled by a Dell computer. These activity chambers identify the animal's position using breaks or disruptions in photocell beams, spaced evenly along the 40 x 40 cm walls of the testing field. A 40 x 40 x 31 cm (l x w x h) Plexiglas box is enclosed within a light and sound-attenuating chamber measuring 53 x 58 x 43 cm. Each box contains a fan that powered on with the system providing ventilation and 'white noise'. At the conclusion of each locomotor session, activity data was collected and translated to the position and/or total distance traveled by each mouse (in cm) in each session by the VersaMax software. For *experiment 2* only, day 0 (Table 2) was a measure of baseline anxiety and locomotor activity, so time spent in the center and time spent in the marginal areas of the box were measured as well.

Experiment 3

Cocaine-induced Locomotor Sensitization

The objective of *experiment 3* was to assess the effects of developmental Pb exposure on cocaine-induced locomotor sensitization in adulthood. The protocol for this behavioral experiment was adopted from Lessov and Phillips (2003) and was a total of 19 days of injections. Similar to experiment 2, each testing day mice were moved into the behavioral testing room 1-hr prior to the start of testing to habituate. All testing occurred under red light as well. On habituation days (1-2), mice received an injection of 0.9% physiological saline (i.p.) and were immediately placed into the Accuscan VersaMax activity monitors for 15 minutes. The baseline locomotor activity for these two days was used to counterbalance mice across three groups: chronic (CC), acute (AC), and saline control (SC). Day 3 was the first day mice in the CC group received injections of cocaine (10 mg/kg; i.p.), while the AC and SC groups received an injection of saline. This continued every other day, with 15-minute activity monitoring until day 11.

Afterward, mice were left undisturbed for one week and a challenge injection was administered on day 19 (expression phase), as shown in Table 3. The SC group received an injection of saline throughout the entire experiment, while the AC group received their first injection of cocaine on day 11 and then a second one on day 19.

Statistical Analysis

All statistical analyses were performed using Prism 8.0 GraphPad Prism Software (San Diego, CA) or SPSS software (SPSS, Version 26, Chicago, IL). While there are no a priori hypotheses about sex, this was a factor included in analyses. When sex was not found to be a significant factor, data were collapsed. In experiment 1, consumption volumes and EPM behaviors (i.e., total arm entries, closed arm entries, open arm entries, head dips, and latency) were analyzed using analysis of variances (ANOVAs) with treatment (Pb or No Pb), sex (male or female), and history (3W or 0W) as between-subject factors and day (only for fluid consumption) as the repeated within-subjects factor.

For both experiments 2 and 3, habituation days were analyzed using a RM ANOVA with treatment and sex as between-subject factors and day as the within-subject factor. The remaining days, or the sensitization phases of each experiment, were analyzed separately. For each experiment, the acute response to the drug was analyzed (day 3) using a three-way ANOVA with sex, group, and Pb exposure as factors. Additionally, for both experiments, analyses of expression day (day 14 or day 11), within-group sensitization (day 3 vs 14 or day 3 vs 11), and across days (days 3-14 or days 3-11) were performed. For experiment 3 only, conditioned actvation and a challenge day was analyzed using a between-group three-way ANOVA with sex, group, and Pb exposure as factors. Groups were split when significant interactions were found and subsequent follow-up ANOVAs and post hoc analyses were used to analyze specific factors within and between groups. Normality tests were performed for all the data, and parametric statistical tests were used as appropriate. Significance for all statistical comparisons was set at $p \leq 0.05$.

RESULTS

Experiment 1

Two-bottle Choice (2BC) Preference Drinking

Across the 21-day two-bottle choice (2BC) drinking paradigm, a RM three-way ANOVA (day x Pb exposure x sex) did not yield any significant interactions; however, there was a main effect of day [$F(4.063, 97.51) = 3.778$, $p= 0.0064$], Pb exposure [$F(1, 24) = 6.603$, $p= 0.017$; Figure 1A – B], and main effect of sex [$F(1, 24) = 14.08$, $p= 0.001$; Figure 1A]. These results suggest that Pb-exposed mice had a higher total EtOH intake than controls and that, overall, females consumed significantly more EtOH than males regardless of pretreatment, which is consistent with previous studies. The average EtOH consumption for Pb exposed animals across days was 16.46 g/kg (F= 19.36 ± 0.40, M= 13.56 ± 0.21), while the control animals averaged 12.78 g/kg (F= 15.26 ± 0.52, M= 10.30 ± 0.55). Moreover, preference for EtOH was assessed next. A RM three-way ANOVA (day x Pb exposure x sex) did not yield any significant interactions; however, there was a main effect of Pb exposure [$F(1, 24) = 7.314$, $p= 0.0124$; Figure 2B], suggesting Pb-exposed mice preferred EtOH at a higher percentage than controls. Next, the water intake for the 3W group was analyzed. Here, there was a significant interaction of day x exposure [$F(8,192) = 2.003$, $p= 0.048$; Figure 3A], suggesting that Pb exposure affected water intake differently depending on the day. Lastly, total water intake from both bottles was analyzed in the 0W group. Overall, there were no significant interactions in total water consumption across the 21-day period ($p> 0.05$); however, there was a main effect of sex [$F(1,24) = 44.29$, $p< 0.001$; Figure 3B], which shows that females consumed more fluid across days regardless of Pb exposure treatment.

Elevated Plus Maze

Following a three-week drinking history, mice underwent an EPM test to assess anxiety-like behaviors. In pilot studies and other literature, Pb exposure has shown to increase anxiety-like behavior (Ferlemi et al., 2014; Shvachiy et al., 2018). Moreover, EtOH consumption and EtOH withdrawal have also shown to increase locomotor and anxiety-like behaviors (Kroener et al., 2012). While we predicted that Pb exposure and a history of EtOH would exhibit the highest anxiety-like

behaviors, that was not the case. Total arm entries (TAE) and closed arm entries (CAE) are crude measures of locomotor activity. Here a three-way ANOVA was performed for all behavioral measures. There were no significant interactions in TAE; however, there was a main effect of sex [$F(1,48)= 4.174$, $p= 0.046$]. Similarly, while there were no significant interaction in CAE, there was a main effect of sex [$F(1, 48) = 4.080$, $p= 0.04$] (Figure 4A – B) and history [$F(1, 48) = 4.759$, $p= 0.03$] (Figure 4B). This revealed that females exhibited greater locomotor behavior than males regardless of history and that a 3W history of EtOH decreased the total number of CAE, which was not expected. Next, the number of open arm entries (OAE) and the open arm time (OAT) or time spent in the open arms were analyzed as percent of the total using a three-way ANOVA. Both OAE and OAT were not affected by Pb exposure or a 3W history of EtOH ($p> 0.05$; Figure 5A – B), yielding no significant interactions or main effects. Interestingly, while we did not see the expected results in the OAE and OAT, there was a main effect of Pb exposure [$F(1, 48) = 9.965$, $p= 0.0028$] and sex [$F (1, 48) = 6.28$, $p= 0.0157$] in latency to enter arm. A Tukey HSD post hoc indicated that the in the 0W animals, Pb-exposed males were significantly different from the No Pb males. Overall, these data suggest that mice exposed to Pb took a longer time to decide which arm to enter than control mice and males also had a significantly higher latency to select an arm as compared to females (Figure 5C).

Experiment 2

Day 0: Open Field

During experiment 2, mice underwent an open field procedure in the same locomotor chamber boxes used for the sensitization experiment. Given the contradicting findings on anxiety-like behavior measured by the EPM we wanted to assess this behavior using a different but well-established model, such as the open field test (OFT). Using standard OFT parameters, mice did not exhibit anxiety-like behaviors, which have been found in the EPM test. Here, using a two-way ANOVA (group x Pb exposure), we found that total boli count, which indicates anxiety and emotionality in mice when elevated (Figure 6A), was not significantly different between groups ($p> 0.05$). Also, using two-way ANOVA (group x Pb exposure), total distance traveled, center distance, and center time was not significantly different across groups as well ($p> 0.05$). This is in contrast to what is consistently found to be true in the literature regarding the OFT (Mattalloni et al., 2017;

Nation et al., 2000); however, it is important to note that the Pb concentration was much higher in these studies.

Habituation

Habituation days 1 and 2 (Figure 7) were used to assess baseline locomotor activity prior to assigning animals to groups. On these two days, an injection of saline was administered before the 15-minute locomotor test. A RM three-way ANOVA (day x Pb exposure x sex) did not yield any significant interactions; however, there was a main effect of day [$F(1, 92) = 37.55$, $p< 0.0001$] and Pb exposure [$F(1, 92) = 11.66$, $p=0.0009$]. Due to a lack interaction or main effect of sex, data were collapsed, and a RM two-way ANOVA was conducted. This analysis revealed a main effect of day [$F(1, 94) = 38.21$, $p< 0.0001$] and Pb exposure [$F(1, 94) = 11.50$, $p= 0.001$]. Further post hoc comparisons using the Sidak test indicated that both Pb exposed and no Pb animals were significantly less active on day 2 compared to day 1 ($p< 0.0001$), which suggests that both groups habituated to the context as expected. Additionally, Pb exposed animals exhibited significantly increased locomotor behavior compared to No Pb animals on days 1 and 2 ($p<0.001$); however, while Pb exposed animals exhibited hyperlocomotive behavior, this finding is in contrast to the locomotor behavior seen during the OFT when an injection of saline was not administered. Due to differences in activity during these two days, mice were counterbalanced by locomotor activity and assigned to one of three groups.

Ethanol-induced Locomotor Sensitization

The complete experiment across days can be seen in Figure 8. Our sensitization protocol paired the context with every injection day (2.5 g/kg EtOH); however, there were no meaningful differences on these days, so they were omitted in further analyses (Figure 8). Before analyzing the key days of ethanol-induced locomotor sensitization (days 3 and 14), the acute response of 2.0 g/kg EtOH was analyzed. The purpose was to see if developmental Pb exposure leads to a heightened locomotor response to this dose of EtOH compared to the group that did not have a Pb exposure period. Day 3 analyzed to assess the acute response in the CE group using a between-group, three-way ANOVA (Pb exposure, group, sex). This analysis found a significant interaction of Pb exposure x group [$F(2, 84)= 3.623$, $p= 0.03$] as well as a main effect of Pb exposure [$F(1, 84)= 13.302$, $p<$

0.0001] and group [f(2, 84)= 3.439, p= 0.037; Figure 9A]. Due to a lack of interactions and main effect of sex, groups were collapsed and followed by a two-way ANOVA. There was an interaction of Pb exposure x group [F(2, 90)= 4.484, p= 0.04] and a main effect of Pb exposure [F(1, 90)= 13.944, p< 0.0001] and group [F(2, 90)=4.429, p= 0.015]. Next, to probe the interaction between Pb exposure and group, the data were split by group first to see if the Pb/CE animals were significantly different than the No Pb controls, which is what we hypothesized. In the CE group, the Pb-exposed animals exhibited an increased locomotor response to the stimulatory properties of EtOH [F(1, 30)= 10.77, p= 0.003]. Next, groups were split by Pb exposure to see if the locomotor response to EtOH in the CE group were different from the activity in the CS and SC groups. There was a main effect of group in the Pb-exposed mice [F(2, 44)= 5.508, p= 0.007]. A Tukey HSD post hoc revealed that the CE groups was significantly different from both the CS and SC groups (p< 0.05), which confirms that this CE group showed an acute response to 2.0 g/kg EtOH. Unsurprisingly, however, in the No Pb groups there was not a main effect of group (p> 0.05), which suggests that the No Pb/CE group did not show a stimulatory response to EtOH on day 3.

Next, activity across the entire 15-minute period on day 3 was analyzed using a RM three-way ANOVA (day x group x Pb exposure). This analysis revealed significant interactions of minute x group [F(18.591, 836.614) = 21.667, p< 0.0001] and group x Pb exposure [F(2, 90) = 4.484, p= 0.014], and a trend for an interaction of minute x group x Pb exposure F(18.591, 836.614) = 1.528, p= 0.071]. Moreover, there was a main effect of minute [F(9.296, 836.614) = 53.699, p< 0.0001], group [F(2, 90) = 4.429, p= 0.015], and Pb exposure [F(1, 90) = 13.944, p< 0.0001; Figure 9B]. Unsurprisingly, when files were split, there was a main effect of Pb exposure in the CE group [F(1, 30)= 10.77, p= 0.003], which corroborates our initial analysis. Over the 15-minute period, the behavior of the No Pb/CE group is as expected, given the mouse strain that was used (Phillips et al., 2011).

To see if animals sensitized within and between groups, days 3 and 14 were analyzed (Figure 10). Files were split by injection group in SPSS in order to determine if Pb exposure impacted sensitization to EtOH in the CE group, specifically. In the CE groups, a RM three-way ANOVA (day x Pb exposure x sex) indicated that there were no significant interactions; however, there was a main effect of day [F(1, 28)= 7.024, p= 0.013] and exposure [F(1, 28)= 4.654, p= 0.04]. Due to no interactions or main effect of sex, groups were collapsed, and a RM two-way ANOVA was used to analyze the treatment group further. This analysis did not yield any significant interactions, but did

indicate a main effect of day [$F(1, 30)= 8.519$, $p= 0.0066$] and Pb exposure [$F(1, 30)= 5.996$, $p= 0.02$]. A Sidak post hoc analysis indicated a significant difference between Pb/CE and No Pb/CE on the acute day of 2.0 g/kg EtOH, but not day 14. Further, post hoc analysis also indicated that, within-groups, there was a significant difference between day 3 and day 14 in the No Pb/CE group, confirming that these animals sensitized to EtOH ($p=0.039$). However, the was not the case in the Pb/CE group, suggesting that these animals did not sensitize to EtOH.

Lastly, an additional method to confirm the sensitization to drugs of abuse is looking at the effects of acute EtOH in the CS group relative to the CE group since their first injection to EtOH occurred on expression day (day14; Figure 11). Thus, a three-way ANOVA (Pb exposure x group x sex) was used to analyze locomotor behavior between groups on day 14. This analysis did not reveal any significant interactions or main effects ($p=0.08$), thus groups were collapsed by sex and followed by a two-way ANOVA. This analysis also indicated that there were no significant interactions and no significant main effects between groups; however, there was a trend for a main effect of group ($p= 0.053$). These results conclude that there were no significant differences between any of the groups on day 14. Thus, the within-group sensitization in the No Pb/CE group was undermined by the fact that the locomotor activity between this group and the No Pb/SC group was not significantly different. In fact, all groups seemed to increase their locomotor across days. Lastly, the CS group was not significantly different from the SC control group, which suggests that when mice are completely habituated to the context, the enhanced acute response seen in the Pb/CE group on day 3 does not occur. Thus, the acute response on day 3 may require a novelty component.

Experiment 3

Habituation

Similar to experiment 2, habituation days 1 and 2 (Figure 12) were used to assess baseline locomotor activity prior to assigning animals to groups. On these two days, an injection of saline was administered before the 15-minute locomotor test. A RM three-way ANOVA (day x Pb exposure x sex) did not yield any significant interactions; however, there was a main effect of day [$F(1, 57) = 87.069$, $p< 0.0001$] and Pb exposure [$F(1, 57) = 13.728$, $p< 0.0001$]. Due to a lack interaction or main effect of sex, data were collapsed, and a RM two-way ANOVA was conducted. This analysis revealed a main effect of day [$F(1, 59) =86.878$, $p< 0.0001$] and Pb exposure [$F(1, 59) = 13.63$, $p<$

0.001]. Further post hoc comparisons using the Sidak test indicated that both Pb exposed and no Pb animals were significantly less active on day 2 compared to day 1 ($p< 0.0001$), which suggests that both groups habituated to the context as expected. Additionally, Pb exposed animals exhibited significantly increased locomotor behavior compared to No Pb animals on days 1 and 2 ($p= 0.001$). Due to differences in activity during these two days, mice were counterbalanced by locomotor activity and assigned to one of three groups.

Cocaine-induced Locomotor Sensitization

The activity data for all groups of this sensitization protocol can be seen in Figures 13-19. Complete activity data for each group across days can be seen in Figure 13. Similar to experiment 2, before analyzing the effect of treatment across days, the acute response to 10 mg/kg cocaine was assessed between groups using a three-way ANOVA (group x Pb exposure x sex; Figure 14). This analysis yielded a significant interaction of group x Pb exposure [$F(2, 49)= 6.554$, $p= 0.003$] and significant main effects of group[($F(2, 49)= 40.975$, $p< 0.0001$], Pb exposure [$F(1, 49)=13.548$, $p= 0.001$]. Groups were collapsed by sex and re-analyzed using a two-way ANOVA. There was a significant interaction of group x Pb exposure [$F(2, 55)= 5.883$, $p= 0.005$] and significant main effects of group [$F(2, 55)= 36.566$, $p< 0.0001$] and Pb exposure [$F(1, 55)=12.056$, $p= 0.001$].

To further probe differences between different groups, files were split by injection group to address whether developmental Pb exposure significantly altered the acute response to 10 mg/kg cocaine compared to No Pb/CC control group. This hypothesis was confirmed [$F(1, 18)=8.639$, $p= 0.009$], suggesting that in the CC group only, the Pb-exposed animals showed a heightened response to an acute injection of cocaine relative to No Pb controls. Moreover, there were no significant differences in either the SAL or AC groups ($p> 0.05$). Next, files were split by Pb exposure to ensure that the acute locomotor response to cocaine was significantly different than the activity level seen in the SAL and AC groups. In the No Pb control groups, there was a main effect of group [$F(2, 28)= 8.052$, $p= 0.002$]. A Tukey HSD post hoc indicated that No Pb/CC was significantly different from both AC and SAL groups ($p< 0.01$; Figure 14), suggesting that there was an increased acute response to cocaine relative to saline controls. Similarly, in the Pb-exposed groups, there was a main effect of group [$F(2, 27)= 30.289$, $p< 0.0001$]. A Tukey HSD post hoc indicated significant differences between Pb/CC and both SAL and AC ($p< 0.0001$). This finding confirms that an acute response to cocaine occurred relative to saline controls. Taken together, these data suggest that developmental Pb

exposure increases the locomotor response to an acute injection of cocaine relative to the No Pb/CC group.

Next, locomotor activity across days 3, 5, 7, and 11 was assessed in order to determine if Pb exposed animals would continue to show a heightened response to 10 mg/kg cocaine (Figure 15). A RM four-way ANOVA (day x group x Pb exposure x sex) revealed significant interactions of day x group [$F(2.659, 130.286)=20.085$, $p< 0.0001$] and group x Pb exposure [$F(2, 49)= 10.211$, $p< 0.0001$]. This analysis also indicated main effects of day [$F(2.659, 130.286)=54.549$, $p< 0.0001$], group [$F(2, 49)= 78.244$, $p< 0.0001$], and exposure [$F(1, 49)= 17.339$, $p< 0.0001$]. Due to a lack of sex effects, groups were collapsed and followed by a RM three-way ANOVA. Here analysis revealed a significant interaction of day x group [$F(5.223, 143.644)= 20.799$, $p< 0.0001$] and group x Pb exposure [$F(2, 55)= 1.064$, $p< 0.0001$]. This three-way ANOVA also indicated main effect of day [$F(2.612, 143.664)=56.332$, $p< 0.0001$], group [$F(2, 55)= 77.6$, $p< 0.001$], and Pb exposure [$F(1, 55) = 17.286$, $p< 0.001$]. A Tukey HSD post hoc analysis of groups indicated that the CC group was significantly different from both the SAL and AC groups ($p< 0.0001$). Thus, further analyses were conducted to assess how they differed in terms of Pb exposure.

First, groups were split by Pb treatment. In the No Pb group (Figure 15A), there was a significant interaction of day x group [$F(5.392, 75.486)= 8.234$, $p< 0.0001$] and a main effects of day [$F(2.696, 75.486)= 30.682$, $p< 0.0001$] and group [$F(2, 28)= 29.6$, $p<0.0001$]. A Tukey HSD post hoc indicated that across all five days, the No Pb/CC group was significantly different than the SAL group. Further, there was a significant difference between the No Pb/CC group and the AC group on days 3, 5, 7, and 9 only. Further, on day 11, the AC group was significantly different than the SAL group ($p= 0.001$). This suggests that while the CC group displayed elevated locomotor activity compared to each group, the AC group showed similar activity to the CC group during their first injection of cocaine. That is a repeated injection of saline resulted in a more heightened acute response to cocaine compared to the repeated cocaine group. In the Pb groups (Figure 15B), there was a significant interaction of day x group [$F(4.859, 65.602)= 13.338$, $p< 0.0001$] and main effects of day [$F(2.43, 65.602)= 26.788$, $p< 0.0001$] and group [$F(2, 27)= 48.593$, $p< 0.0001$]. A Tukey HSD post hoc indicated that across all five days, the Pb/CC group was significantly different from both the AC and SAL groups, suggesting this increased activity level due to repeated exposure to the drug. Next, these data were split by injection group to probe differences between Pb exposure activity in the CC groups specifically. There was a trend for an interaction of day x Pb exposure [$F(4, 72)=$

2.141, p= 0.08] and main effects of day [F(4, 72)= 23.589, p< 0.0001] and Pb exposure [F(1, 18)= 14.426, p= 0.001]. A Sidak post hoc indicated that the Pb exposed animals showed an increased response to 10 mg/kg cocaine compared to the No Pb controls across all five days (all p< 0.04; Figure 15C), which confirms our initial hypothesis. That is, low-level Pb exposure alters the locomotor response to cocaine across days.

Classically, sensitization is defined as an increased response to a fixed dose across days. The difference between day 3 and day 11, specifically, will allow us to confirm whether there was within-group sensitization (Figure 16). Day 3 versus day 11 were evaluated separately to test within and between-group sensitization in the CC and AC groups. First, files were split between groups, to look at within-group sensitization in the CC groups. A RM three-way ANOVA (day x Pb exposure x sex) revealed no significant interactions; however, there was a main effect of day [F(1, 16)= 112.562, p< 0.0001] and Pb exposure [F(1, 16)= 10.55, p= 0.005]. This suggests that regardless of day, Pb exposed animals in the CC group showed significantly higher locomotor activity compared to the No Pb controls on days 3 and 11. Thus, within-groups, both the Pb/CC and the No Pb/CC groups sensitized to 10 mg/kg cocaine, but to a different degree. Unsurprisingly, the SAL animals did not show any main effects of day or Pb exposure (p> 0.05), which suggests that the initial hyperactivity seen during habituation is diminished as days went on. Due to lack of interactions or main effects of sex, groups were collapsed and followed up using a RM two-way ANOVA. Here, there were no significant interactions. There was a main effect of day [F(1, 18)= 100.317, p< 0.0001] and Pb exposure [F(1, 18)= 10.44, p= 0.005]. A Sidak post hoc analysis indicated differences between day 3 and day 11 in both CC groups, which suggests that both CC groups sensitized to 10 mg/kg cocaine (p< 0.0001). Further, between treatment groups, Pb and No Pb/CC were significantly different from one another on day 3 and day 11 (p< 0.01). This finding indicates between-group sensitization and that developmental Pb exposure increases the stimulatory effects of cocaine compared to No Pb controls.

As mentioned briefly in experiment 2, an additional method to confirm sensitization is to compare chronic groups to an acute group receiving their first injection of the drug on the last day. Here we hypothesized that Pb and No Pb/CC groups should exhibit increased locomotor activity compared to both saline and acute groups. To assess differences between groups on expression day (day 11) a three-way ANOVA (group x Pb exposure x sex) was used (Figure 17). This analysis did not yield any significant interactions; however, there was a main effect of Pb

exposure [F(1, 49)= 5.329, p= 0.025] and group [F(2, 49)= 43.143, p< 0.0001]. Groups were collapsed by sex and followed by a two-way ANOVA. This analysis did not yield any significant interactions (Pb exposure x group; p= 0.07), but there was a main effect of Pb exposure [F(1, 55)= 5.77, p= 0.02] and group [F(2, 55)= 46.559, p< 0.0001]. To further probe these effects, groups were split by Pb exposure and group. First, when split by Pb exposure, a Tukey HSD post hoc indicated significant differences between the SAL and AC groups (p= 0.001) and the SAL and CC (p< 0.0001) groups. The CC and AC groups were not significantly different from one another (p= 0.19). In the Pb groups, a Tukey HSD post hoc indicated a significant different between the SAL and AC groups (p= 0.001), the SAL and CC groups (p< 0.0001), and the CC and AC groups (p= 0.002). A between-group analysis indicated a significant difference between the Pb/CC and No Pb/CC groups [F(1, 18)= 9.683, p= 0.006], but there were no significant differences in the other two groups.

Following expression day, all subjects received a saline injection (day 12). This day reflects conditioned activation or the effects of repeated injections of cocaine on baseline locomotor activity. This is defined as day 12 minus habituation day 2, which can be seen in Figure 18. A univariate ANOVA did not yield any significant interactions; however, there was a main effect of group [F(2, 49)= 8.645, p= 0.001]. Due to a lack of sex effects, the data were collapsed. An ordinary one-way ANOVA indicated a main effect of group [F(2, 55)=9.489, p< 0.0001]. A Tukey HSD post hoc indicated a significant difference between the Pb/CC group and the Pb/SAL group (p= 0.0017), suggesting cocaine-induced conditioned activity in the Pb/CC group only.

A challenge injection of cocaine or saline was given one week later on day 19 (Figure 19). The locomotor responses between groups were analyzed using a three-way ANOVA (group x Pb exposure x sex). There were no significant interactions (group x Pb exposure, p=0.08); however, there was a trend for a significant three-way interaction of group x Pb exposure x sex (p= 0.056), which appears to be driven by the AC groups (Figure 19A). Additionally, there was a main effect of group [F(2, 49)=38.262, p< 0.0001]. There were no a priori hypotheses about sex effects. Thus, while this interaction is trending toward significance, groups were collapsed, and a two-way ANOVA was conducted. This analysis did not show any significant interactions, but there was a main effect of group [F(2, 55)= 35.103, p< 0.0001]. Further, a Sidak post hoc analysis indicated that within the No Pb groups, the CC and AC groups were significantly different than

the SAL group ($p< 0.001$); however, there was no significant difference between the CC and AC groups ($p> 0.05$; Figure 19B), which is a similar pattern to what was observed on day 11. In the Pb groups, there was a significant difference between the CC group and both the AC and SAL groups ($p< 0.001$). Additionally, there was a significant difference between the AC group and the SAL group ($p= 0.005$). Again, these results are similar to the results found on day 11. One of our a priori hypotheses was that Pb-exposed animals would continue to show an increased response to chronic cocaine after a withdrawal period. Thus, a Sidak post hoc test was used to see if there were difference between Pb/CC and No Pb/CC groups. Here there was a significant difference between the two groups ($p= 0.04$), which confirms that even after a withdrawal period, animals developmentally exposed to Pb continue to show an increased locomotor response to 10 mg/kg cocaine compared to No Pb controls with a history of repeated injections.

Lastly, to confirm whether increased activity persisted across days, a four-way RM ANOVA (day x group x Pb exposure x sex) was conducted for acute, expression, and challenge days (3, 11, 19; Figure 20). Analyses indicated significant interactions of day x group [$F(3.529, 86.471)= 16.779$, $p< 0.0001$] and group x Pb exposure [$F(2, 49)= 4.267$, $p= 0.02$]. There were also significant main effects of day [$(1.765, 86.471)= 101.368$, $p< 0.0001$], group [$(2, 49)= 50.317$, $p< 0.0001$], and Pb exposure [$F(1, 49)= 6.693$, $p= 0.013$]. There were no significant interactions or main effects of sex, thus groups were collapsed and followed with a three-way ANOVA. This analysis revealed significant interactions of day x group [$F(3.492, 96.031)= 16.081$, $p< 0.0001$] and group x Pb exposure [$F(2, 55)= 4.121$, $p= 0.021$]. Moreover, there were main effects of day [$F(1.746, 96.031)= 95.591$, $p< 0.0001$], group [$F(2, 55)= 48.899$, $p< 0.001$], and Pb exposure [$F(1, 55)= 6.519$, $p= 0.013$]. To further probe the interactions with respect to the CC groups, files were split by injection group. In the CC groups, there was a main effect of day [$F(2, 36)= 69.702$, $p< 0.0001$] and Pb exposure [$F(1, 18)= 8.49$, $p= 0.009$]. A Tukey HSD post hoc indicated significant difference between day 3 and day 11 as well as day 3 and day 19 in both Pb/CE and No Pb/CE groups ($p< 0.0001$). There was also a significant difference between Pb and No Pb/CC groups across days ($p< 0.05$). While day 11 and day 19 did not differ statistically ($p> 0.05$), day 19 activity was significantly different than the activity seen on day 3, suggesting that sensitization persisted after a one-week withdrawal period.

DISCUSSION

Summary of Findings

Three different experiments were conducted to elucidate if translational BLLs during the developmental period (BLL <6 μg/dL) alter drug responses in adult mice. Chronic, low-level Pb exposure from PND 21- PND 42 resulted in increased EtOH consumption and preference as well as a Pb/EtOH interaction by which latency to select an arm in the EPM was decreased. Animals with developmental Pb exposure did not show increased anxiety-like behaviors as measured by the open field test (OFT); however, they did exhibit hyperlocomotor activity when administered an injection of saline and an elevated acute response to 2.0 g/kg EtOH. Moreover, repeated EtOH injections resulted in a modest sensitization response in the No Pb/CE group only; however, these findings were undermined by the fact that the activity level seen in the saline control animals was not significantly different than the No Pb/CE group on expression day. Additionally, developmental Pb exposure increased the acute response to 10 mg/kg cocaine in the CC group compared to the non-exposed controls and resulted in an overall heightened sensitization response across days as compared to the non-exposed chronic group. Lastly, there was also evidence that sensitization to cocaine persisted following a one-week withdrawal period in both CC groups and that Pb exposure continued to enhance the locomotor response compared to non-exposed animals; however, there were no significant differences between day 19 activity and day 11.

Aim 1

Developmental Lead Exposure Increases EtOH Consumption and Preference

The objective of experiment 1 was to determine if chronic low-level Pb exposure during development would influence EtOH consumption and preference in adulthood and whether a Pb/EtOH interaction would influence their anxiety-like behavior on the EPM. We hypothesized that Pb exposure would increase EtOH consumption and preference in a 2BC drinking paradigm, which was confirmed. Moreover, Pb exposure did not affect water intake in the Pb/0W animals, suggesting that these differences are specific to EtOH and not overall fluid consumption. We

also hypothesized that a history of EtOH would result in anxiolytic effects. Specifically, the Pb/3W (EtOH) group would exhibit behaviors similar to controls, while the Pb/0W (no EtOH) group would exhibit enhanced anxiety-like behaviors on the EPM compared with the No Pb/0W control group. The drinking results are in line with what has been seen in both the human literature (Morisi et al., 1989; Pizent et al., 2001) and studies conducted in rats exposed to toxic levels of Pb (Mattalloni et al., 2017; Virgolini et al., 2019; Virgolini & Cancela, 2014). However, this is the first study to show that mice, with a propensity to drink EtOH, will voluntarily consume more than control animals at a consistent 10% v/v EtOH solution as well as show an increased preference for EtOH (Figure 1 & 2). Further, in experiment 1, we single-housed animals once they reached adult age, which allowed for an assessment of how each mouse consumed across days. This is in contrast to the few studies that have focused on this research. Other preclinical drinking studies have used pair-housed 2BC models, which include presenting four fluid tubes for preference testing (Virgolini et al., 2019; Virgolini & Cancela, 2014), making it difficult to identify the exact fluid consumption of each animal.

Further, C57BL/6 mice are an inbred mouse strain that readily consume EtOH to pharmacologically relevant levels. While other studies have shown an increase in consumption, their methodologies differed and required increasing the EtOH concentration across days until they reached 10% v/v in order to ensure that the rats would consume high volumes. Moreover, C57BL/6 mice are commonly tested in Drinking-in-the-Dark (DID) models of alcohol binge drinking. In experiment 1, a 2BC preference drinking paradigm was selected to assess the effects of Pb exposure on voluntary consumption because previous findings from our lab found that there were no differences in binge-like drinking between the treatment groups using a DID protocol (unpublished). These results could be due to differences in metabolism or the ability to titrate with water in the 2BC protocol. While 2BC is a 24-hour model, DID only allows for a two-hour daily drinking period.

Here we did not focus on the mechanism of action for these behavioral changes; however, Pb and EtOH have a close relationship. Several studies have found that EtOH modifies the distribution and mobilization of Pb by affecting the membrane permeability of all cells (Flora et al., 2012; Virgolini et al., 2019). This, in turn, facilitates Pb gastrointestinal absorption, which increases the brain Pb levels and promotes the toxic effects (Virgolini et al., 2019). While these animals were no longer ingesting Pb, one possibility could be that there may have still been Pb

stored in bone tissue, which could have traveled back into the bloodstream and penetrated the brain. Further, EtOH has reinforcing properties facilitated by DA neurotransmission. Given that Pb alters the reinforcing properties of EtOH, several reports have focused on EtOH, brain acetaldehyde (ACD), and salsolinol collectively, which together mediate the increased response in DA neurotransmission. Briefly, ACD, which is promoted via blood catalase (CAT) and removed via ALDH2, is a common site of action for EtOH and Pb (Virgolini et al., 2017). Thus, understanding how or if EtOH metabolism is disrupted could provide novel insight into how Pb exposure at different stages of life alters appetitive behaviors.

Effects of Lead Exposure on Anxiety-like Behaviors After 24-hr Withdrawal

Next, we tested animals on the EPM to see if there would be an interaction between a history of EtOH consumption and Pb exposure that would alter anxiety-like behaviors. Previous findings from our lab suggested that C57BL/6 mice exposed to Pb show increased anxiety-like behaviors on the EPM test without an EtOH history (unpublished), which is consistent with the current human and preclinical literature (Leret et al., 2003; Shvachiy et al., 2018; Sobin et al., 2013; Virgolini et al., 1999). In order to avoid stress, no injections of EtOH were given. Thus, it should be noted that mice were not intoxicated at the time of the test. Instead, we were probing the effects of a three-week EtOH history, which can also be addressed as a 24-hour EtOH withdrawal period, on the EPM. One of the hypotheses was that there would be an increase in anxiety-like behaviors in the Pb/0W group compared to the No Pb/0W group; however, there were no differences between the Pb-exposed and No Pb groups without an EtOH history in any of the anxiety-like measures. Given the previous findings from our lab (unpublished) and others (Shvachiy et al., 2018) showing Pb exposure increases anxiety-like behaviors, these inconsistent findings in our EtOH naïve groups were most likely a result of other methodological differences, such as being single-housed for three weeks versus being group-housed prior to the test. Specifically, studies have found that single-housed mice show increased locomotion and decreased anxiety-like behaviors on the EPM test (Võikar et al., 2005). Moreover, there is no information about how or if developmental Pb exposure alters anxiety-like behavior during specific ages. Future studies are needed to address how persistent anxiety-like behaviors are after developmental Pb exposure using consistent methodologies.

In the EPM test, total arm entries and closed arm entries are a crude measure of locomotor behavior. After a three-week EtOH history, there were no differences in total arm entries based on history or exposure, but findings showed that females entered more arms than males, which is consistent with the literature showing that females generally show higher locomotor activity than males (Tucker & McCabe, 2017). Moreover, there was a main effect of EtOH history and sex on the number of closed arm entries, which is where mice tend to stay when they are more anxious. These findings suggest that a history of EtOH decreased overall locomotor activity and that females were generally more active than males, which is consistent with current literature (Kliethermes, 2005; Tucker & McCabe, 2017).

Further, there were no significant differences between history or treatment groups in the number of open arm entries and the time spent in the open arms as a percent of the total arm entries (Figure 5A-B). Lastly, there were differences in the latency to select the first arm on the EPM (Figure 5C) where males, in general, took longer deliberating in the center of the maze before making an initial decision than females. Further analyses concluded that there was also a main effect of Pb exposure where, in both sexes, Pb-exposed groups without a history of EtOH took longer deliberating where to go than any of the other groups, suggesting that a history of EtOH rescued the anxiogenic properties of Pb exposure in this behavior, which was not expected. While there is no clear explanation, it is important to consider additional factors as the effects of both Pb and EtOH are multisystemic. Specifically, oxidative stress enhances the detrimental effects of Pb/EtOH interactions (Flora et al., 2012; Virgolini et al., 2019) and EtOH increases the effects of Pb as well (Correa et al., 1999).

Developmental Lead Exposure May Effect males and Females Differently

While there were no a priori hypotheses about potential sex differences, future studies should focus on probing this specific question as some studies have suggested that there are indeed sex-specific differences on how Pb exposure alters the brain and behavior (Cecil et al., 2008; Singh et al., 2018; Sobolewski et al., 2018; Tena et al., 2019). Cecil et al. (2008) found decreased gray matter in several brain regions in adults with a history of Pb exposure during childhood and that this decrease was more pronounced in males compared to females. This is consistent with other studies that have found males are more sensitive to the effects of Pb with respect to behavior (Singh et al., 2018; Wright et al., 2008). In contrast, Tong et al. (2000) found

that females were more sensitive to the effects of Pb exposure, showing that IQ was more effected than males. Findings have been inconsistent across studies, largely due to differences in populations tested and the behaviors chosen to correlate with Pb exposure. Moreover, it is now known that time and length of exposure (pre- vs postnatal) is important to consider. Further, there are relatively few preclinical studies that have used both sexes in their studies. Thus, there is limited information on how Pb exposure affects males and females differently; however, with the few studies that exist, differences between males and females have been identified with respect to anxiety (anxiogenic effect in males only) (Ajarem & Abu-Taweel, 1992; Soeiro et al., 2007) and olfactory memory recognition (only males impaired) (Flores-Montoya et al., 2015). Across the preclinical literature, similar to human studies, there are limitations. Inconsistencies throughout the literature are largely due to differences in species and methodologies used. Here we did not find any sex x Pb exposure interactions with respect to the EPM. However, given the fact that others have seen sex differences in behavioral tests, future research should consider this moving forward.

Aim 2

Open Field Test

In experiment 2, no group sensitized to the stimulatory effects of EtOH as defined by the literature. Additionally, our anxiety-like measures were not replicated using the OFT as hypothesized. Prior to the sensitization protocol, animals were tested using a 20-minute OFT on day 0 (Figure 6). During the test, there were no differences in boli count, center distance, or center time between the Pb-exposed and control animals, which are the biggest measures of anxiety-like behavior in this test. Further, there were also no differences in total locomotor activity (Figure 6B), which is surprising as most of the literature has consistently found that Pb-exposed rodents exhibit hyperlocomotion as compared to control animals (Ma et al., 1999; Mattalloni et al., 2017; Nation et al., 2000; Zuch et al., 1998). Further, both the EPM and OFT are inherently stressful behavioral paradigms (Nosek et al., 2008); however, the type and degree of stress is different, which might explain why these findings did not replicate. While the OFT may measure baseline stress due to the novelty of the box, in the EPM there is stress due to the novelty of the apparatus in addition to the height of the apparatus, which may induce

physiological stress (Nosek et al., 2008). One thing to consider is that this experiment has animals that were pair-housed and thus were treated identically to the group previously tested on the EPM test, which suggests that these tests may measure fundamentally different types of stress and anxiety (Nosek et al., 2008).

Effects of Lead Exposure on Ethanol-induced Behavioral Sensitization

Next, mice underwent habituation to the testing chambers (day 1-2), which also included an injection of saline on each day immediately prior to the 15-minute test. This was to determine if there were any underlying basal locomotor differences and to acclimate the animals to the daily injection procedure. Interestingly, mice previously exposed to Pb exhibited increased locomotor activity as compared to the No Pb controls (Figure 7). The differences persisted on day 2; however, both groups declined in their overall locomotor activity as compared to day 1, which corroborates previous findings. While all animals habituated to the testing chamber, Pb-exposed mice continued to exhibit an increased locomotor response. As mentioned before, Pb exposure is known to increase locomotor behavior; however, in this experiment, hyperlocomotion only occurred when paired with an injection. In previous studies, where hyperlocomotion was found in the OFT, animals were exposed to much higher levels of Pb (Ma et al., 1999); however, there are no studies comparing the levels that were used in our experiment. While the methodologies are not identical, Virgolini et al. (2004) found similar results in adolescent rats, but not adults. In their study the authors found an increased locomotor response and increased corticosterone secretion in PND 35, Pb-exposed rats when a saline injection was administered after habituation in a novel context; however, the habituation period (no injection) in this experiment was 30 minutes prior to the injection rather than one day apart. Interestingly, they did not see an increased response a mild stressor in PND70 rats, which is in contrast to what we found here.

One reason for the differences in activity found between the OFT and habituation days could be explained by a Pb/stress interaction if Pb was still present at the time of testing, as briefly mentioned in experiment 1 (Virgolini et al., 2004, 2019). There is growing evidence that stress exacerbates the neurotoxic effects of Pb (Rossi-George et al., 2011). For example, Graham et al. (2011) found that Pb-exposed animals had elevated levels of homovanillic acid (HVA), which often serves as a marker of DA neuronal function. Thus, the stress induced by the

injection could have resulted in hyperlocomotor activity as a result of the interaction of Pb and stress on DA neurotransmission (Graham et al., 2011). It is important to note that here we did not measure Pb levels in these animals. Thus, we cannot confirm that there was Pb in the system at the time of testing. An alternative explanation is that developmental Pb exposure altered the maturation process of the hypothalamic–pituitary–adrenal (HPA) axis and/or the DA system disrupting normal functionality in adulthood (Virgolini et al., 2004; White et al., 2007). A study conducted by Cory-Sletcha et al. (2004) found increased basal levels of corticosterone in adult male and female rats with a history of maternal Pb exposure. These data are compelling and provide evidence of permanent changes to HPA axis; however, the mechanism by which this occurs is not entirely know. Specifically, it is not known if this is a direct result of Pb exposure or indirectly, through Pb-associated changes in the mesocorticolimbic systems (Barrot et al., 2000; Deborah A. Cory-Slechta et al., 2004; Moghaddam, 2002).

On day 3, the CE group experienced their first dose of 2.0 g/kg EtOH (Figure 9). As predicted, the Pb/CE group exhibited an increased acute response to EtOH; however, there was no difference between No Pb/SC and No Pb/CE groups, which means that the stimulatory effects of EtOH were not expressed by this group. This is not surprising as most models of EBS using C57BL/6 mice show little to no response to this dose of EtOH (Bosse et al., 2019; Crabbe & et al, 1982; Phillips et al., 2011). Interestingly, the acute response exhibited by the Pb/CE group on day 3 did not replicate in the Pb/CS group (acute) on expression day (day 14), which suggests that novelty may have played an important role in the acute response to EtOH seen in Pb/CE group (Figure 11). The CS groups were repeatedly injected with saline, taking away any novelty aspect of the chambers as a result of extended habituation, prior to their first injection of EtOH.

Moreover, the key test days for defining EBS were day 3 and day 14; however, in this experiment, the context was paired, allowing for an analysis to be conducted for days 3-14 as well. On the days 4-13 (Figure 8), where the CE group receive daily injections of 2.5 g/kg EtOH and the other two groups (CS, SC) received saline, there were no overall meaningful locomotor differences between or within groups. Thus, these days were excluded from further analyses. Through an analysis between day 3 and day 14, our findings indicated that C57BL/6 mice without pretreatment of Pb exposure sensitized to repeated injections of EtOH, while the Pb/CE did not (Figure 10); however, this finding in the No Pb/CE group was undermined by the fact that there was no difference in locomotor activity between this group and the No Pb/SC and No

Pb/CS animals on expression day (day 14) (Figure 11). Thus, though significant, there was no within-group or between-group sensitization regardless of Pb exposure. Interestingly, the lack of difference between the No Pb/CS group and the SC group indicated that there was also not a significant acute response to EtOH in this group. Overall, these findings suggest that while Pb-exposure did increase the acute response to 2.0 g/kg EtOH, this does not mean that there was an increased sensitivity to EtOH; however, there were several limitations with the current study, and with the mouse genotype used to test EBS.

Ethanol-induced Behavioral Sensitization and Variability Across Genotypes

One reason for the lack of sensitization observed in the CE groups could be that context can impact the efficacy of the drug sensitization, suggesting that novelty may be important for learning and expressing the sensitivity to EtOH in certain genotypes (Boehm et al., 2008; Castello et al., 2015). For example, there is evidence that sensitization resistant C57BL/6J mice, which were used here, do not sensitize or sensitize poorly when EtOH is repeatedly administered in the same context (Boehm et al., 2008; Phillips et al., 2011). Thus, the EBS methodology for this strain differs from protocols used for other genotypes. In contrast, the most commonly used mouse strains for EBS are the DBA/2J (inbred) and the Swiss Webster (outbred) strains. These mice tend to be more reliable sensitizers and show more robust sensitization whether the context is paired or not.

There have been contrasting findings on the relationship between EtOH intake and EBS across mouse models. Some studies have found that there is no difference in consumption volumes between “high-” and “low-sensitizing” mice; however, several studies have found that there are differences between genotypes with opposing alcohol preferences (Camarini et al., 2011; Phillips et al., 2011). More specifically, mouse strains that voluntarily consume high volumes of alcohol, such as the C57BL/6J, either do not exhibit or exhibit minimal sensitization to EtOH (Boehm et al., 2008; Phillips et al., 2011). In contrast, mouse strains that do not voluntarily consume EtOH and find the taste of EtOH aversive, such as the DBA/2J mice, display high sensitization to EtOH (Boehm et al., 2008; Didone et al., 2019; Legastelois et al., 2014; Phillips et al., 2011). However, it is not as simple as this. Specifically, selectively bred high alcohol preferring (HAP) mice do show an increased locomotor response to EtOH after repeated injections, but not the low alcohol preferring (LAP) mice (Grahame et al., 2000). These

data are consistent with findings from Newlin and Thompson (1990). In this study, the authors found that sons of parents with a history of alcohol use disorder (AUD) form an acute sensitization compared to sons of parents without AUD (Newlin & Thomson, 1990).

Lessov et al. (2001) investigated this relationship more closely in these inbred strains by having mice drink before or after a sensitization protocol. They found that following a sensitization protocol, C57BL/6J mice increased their voluntary consumption of EtOH compared to saline controls, while DBA/2J mice showed no change in voluntary consumption following sensitization. Also, researchers explain that this heightened locomotor response to EtOH in C57BL/6J was only seen in groups with a pre-exposure to EtOH. This evidence is compelling and suggests that genetics and history of alcohol consumption may contribute to an individual's ability to sensitize to EtOH, but also that EBS may not be associated with a greater avidity for EtOH as seen in the DBA/2J data (Lessov et al., 2001). Thus, it may be beneficial for future studies to investigate the effects of developmental Pb exposure and sensitization across different genotypes with varying sensitization effects.

In our model, the context was paired with each injection because Pb exposure has shown to cause cognitive deficits (Fishbein et al., 2008). In EBS, these long-lasting neuroadaptive changes occur via long-term potentiation (LTP), which is a form of neuroplasticity. LTP is defined as the strengthening of synapses as new information is processed by repeated stimulation of two neurons at the same time (Lovinger & Kash, 2015; Rochester, 2009). Drugs of abuse alter naturally- occurring synaptic function and LTP by enhancing excitatory neurotransmission. Most of this cell excitation is seen in the ventral tegmental area (VTA) via dopaminergic receptors and NMDAR activation (Rochester, 2009). Here we wanted to see if learning deficits would arise in this behavioral test, such that Pb-exposed mice would have difficulty habituating to the context and therefore lead to a hypersensitivity to the EtOH. However, this was not the case. As a result, we did not probe days 4-13 because there were no differences in locomotor activity during the induction phase between the Pb and No Pb CE groups during our statistical analyses.

This is the first experiment to look at the effects of developmental Pb on EBS in any rodent model. Given that there was a difference in the acute response to 2.0 g/kg EtOH on day 3 (Figure 9A – B), future studies should replicate our model and probe whether perinatal Pb exposure alters EBS without pairing injections to the context. Several preclinical studies have shown that C57BL/6J mice will modestly sensitize to EtOH when the context is not paired. Thus, we cannot

conclude that Pb-exposure does not alter EBS because our control CE group did not form a sensitization to EtOH as there was no difference between this group and saline controls on day 14.

Aim 3

Effects of Lead Exposure on Cocaine-induced Behavioral Sensitization

For the following experiment, the sensitization protocol was adapted from Phillips et al. (2011); however, here we added a one-week withdrawal period, and mice were left undisturbed until day 19, where they received a challenge dose of 10 mg/kg cocaine. The initial habituation days, as discussed in experiment 2, resulted in a replication of the behavioral outcomes. Mice in experiment 3 exhibited increased basal locomotor activity during habituation days 1 and 2 as compared to controls (Figure 12), which was expected.

Based on the known mechanisms of action, we hypothesized that chronic developmental Pb exposure would increase the locomotor effects of 10 mg/kg dose of cocaine as compared to controls, which was confirmed. The Pb/chronic cocaine (CC) group exhibited an increased response compared to control animals across days 3, 5, 7, 9, and 11, resulting in a between-group difference in sensitization (Figure 15 A – C; Figure 16).

One factor that provides evidence of within-group sensitization is the difference or change in activity level between key days 3 and 11 (Phillips et al., 2011). In both CC groups, there was a significant difference between days 3 and 11, which is evidence of within-group sensitization (Figure 16). A dose of 10 mg/kg has been shown to cause robust sensitization in C57BL/6 mice, and previous studies have shown activity levels similar to what was seen in this experiment (Phillips et al., 1998). While there was only one dose tested in this experiment, others have looked at the effects of Pb exposure at higher doses, and there is one study that has looked at the effects of Pb on lower doses of cocaine (Virgolini & Cancela, 2014). Unpublished data from Virgolini and Cancela (2014) showed that low-level Pb exposure increased the locomotor response compared to control animals at a dose of 5 mg/kg cocaine but did not cause an increase in activity in either group at 2.5 mg/kg. These data are compelling and suggest that Pb exposed mice may be able to sensitize to cocaine at lower doses, while control animals do not. Future

studies should be conducted in order to assess whether developmental Pb exposure, specifically, leads to increased activity at 5 mg/kg and at lower doses of cocaine.

Contrary to our findings, Nation et al. (1996) found that BLLs >25 μg/dL attenuated cocaine sensitization in the Pb-exposed groups. However, this study was conducted in rats, and Pb-exposure began in adulthood and continued throughout the entirety of the experiment. The differences between their findings and the supersensitivity, or increased sensitivity compared to baseline sensitivity, seen in the current study suggest that these changes may be specific to the age of Pb exposure or the Pb concentration. Further, Nation et al. (2001) also conducted a similar experiment and saw an attenuation in locomotor activity in adolescent and adult rats, but only during the acute response of 10 mg/kg cocaine. After the initial injection, subsequent activity showed that developmental Pb exposure at 16 mg Pb (yielding BLLs > 12 μg/dL) showed a supersensitivity to cocaine. Literature has shown that behavioral differences can be specific to the time, length, and amount of exposure. Due to their elevated BLLs, the attenuation of locomotor activity could have been attributed to the high Pb levels, since it is known that high levels of Pb decrease overall DA activity while low levels increase it (Deborah A. Cory-Slechta & Widzowski, 1991; Jones & Miller, 2008). An explanation for the attenuated response could be that high levels of Pb could be blocking the release of DA in the synapse, thereby allowing less DA to be available for reuptake by DAT, which is important for the stimulatory properties of cocaine (D. A. Cory-Slechta, 1995).

Further, sensitization was also assessed between all group conditions on expression day (day 11) to see if the activity level of repeated injections of cocaine differs from the acute groups (Figure17). On day 11, the Pb/CC group showed significantly higher locomotor activity compared to No Pb/CC as well as higher activity than the Pb/AC group (Figure 17). This finding confirms that Pb groups sensitized as defined by the current literature. Further, in the No Pb groups, the response from the AC group was not significantly different from the No Pb/CC group ($p > 0.05$) (Figure 17). Instead, the acute response from the AC group was greater than the acute response on day 3 from the CC group. This is interesting and could suggest that repeated injections of saline changed the behavior of the acute response to 10 mg/kg cocaine, which has been seen in other studies (Phillips et al., 1998).

To reassess conditioned locomotor activity, mice received one injection of saline immediately prior to a 15-minute test. Thus, day 12 was also analyzed separately. There was

evidence for associative conditioning effects as cocaine sensitization was found in the Pb-exposed animals, but not the controls. The Pb/CC group showed significantly higher activity than Pb/SC animals, but not the Pb/AC animals. In the No Pb groups, there was no evidence of conditioned effects in any group (Figure 18). Differences could be attributed to the time and amount of cocaine injections prior to the injection of saline. Specifically, testing each group for conditioned activation 24 hours later could have influenced activity as subjects had not received consecutive injections prior to this test. In EtOH sensitization experiments, conditioned activation is variable as there is evidence for and against it (Cunningham, 1995; Phillips et al., 1997)

Effects of Lead Exposure on Cocaine Sensitivity After One-Week Withdrawal

Last, mice received a challenge injection of 10 mg/kg cocaine on day 19, one week following their last injection. Drug-induced behavioral sensitization (DBS) has shown to persist long after the expression phase (Boehm et al., 2008; Phillips et al., 2011). For cocaine sensitization specifically, one study found that sensitization persisted as long as two months following the last injection of a 20 mg/kg dose (Shuster et al., 1977), while a separate study in rats found that sensitization persisted for one month after a 10 mg/kg dose (White, Hu, Henry, & Zhang, 1995). Given the current literature and what is known about the effects of Pb on cocaine sensitivity, it was hypothesized that Pb-exposed mice in the CC group would continue to show a heightened sensitization response following a one-week withdrawal period compared to the No Pb/CC and both AC groups. On day 19, the No Pb/CC group was not different from the No Pb/AC group, but there was a difference between the CC and AC groups in the Pb-exposed animals on day 19 (Figure 19). In a separate analysis, it was concluded that day 19 was not significantly different from day 11 (Figure 20) in any of the CC or AC groups but was different from the acute response (day 3) in all, but the SC groups. While there were no significant differences between day 11 and day 19 in any group, most groups showed a slight increase in locomotor activity, which is consistent with what is seen in other studies with a one-week withdrawal period (White et al., 1995). The only group that did not seem to show a visible locomotor increase was the Pb/AC group, suggesting that Pb may have underlying effects on locomotion following a one-week withdrawal period. Moreover, while there was no difference between these key days (11 & 19), both Pb and No Pb/CC groups were significantly different

from the control groups on day 19, which was hypothesized. Of the few studies that have looked at the effects of Pb exposure on cocaine sensitization, none have assessed challenge injections after a withdrawal period, and both studies from Nation et al. (2000, 2003) gave consecutive injections, which has shown to alter the behavioral response to the drug (Phillips et al., 2011). Ultimately, this is the first experiment to see if Pb exposure continues to show enhanced activity a week after the last injection, which was confirmed.

Mechanism of Findings in Drug-induced Behavioral Sensitization

While the mechanisms of action were not explored in these experiments, it is widely known that dopamine (DA) plays a crucial role in the rewarding properties of drug consumption and drug-induced locomotor sensitization. It is also known that chronic Pb exposure alters DA synthesis and release in different brain regions, which seems to be attributed to the disruption of presynaptic mechanisms (D. A. Cory-Slechta, 1995; Ma et al., 1999; Zuch et al., 1998). Further, there is also evidence for altered DA metabolites (DOPAC and HVA) and increased DA turnover, or the ratio between DA metabolites and DA, in the striatum following Pb exposure via gestation and through PND 50 (Stansfield et al., 2015). However, whether Pb acts directly on DA or indirectly is yet to be determined. In experiment 2, we evaluated the effect of developmental Pb on EtOH-induced behavioral sensitization (EBS). Unlike most drugs that induce stimulatory behavioral sensitization, EtOH has both stimulatory and sedative effects (Didone et al., 2016). In this respect, the stimulatory effects of EtOH in EBS seem to be mediated by the effect that EtOH has on the dopaminergic system.

There are two different classes of receptors: D1-like receptors and D2-like receptors. The D1 subtype is made up of D1 and D5, which are Gs protein-coupled receptors, and the D2 subtype is made up of D2, D3, and D4, which are Gi protein-coupled receptors (Camarini & Pautassi, 2016). The literature is clearer and more consistent surrounding the contributions of the D1 receptor pathway in the NAc to the development of EBS. Early publications have shown that D1 antagonism, systemically or intra-NAc, blunted both the induction and expression phases of EBS (Abrahao et al., 2011), which suggests that D1 receptor mechanisms are important in mediating the effects of EtOH. The inconsistencies seen in studies looking at D2 receptors may be as a result of the drug compounds used. Some D2 agonists and antagonists act on both D2 and

D3 receptors, which can have different effects on behaviors. More specifically, EtOH has shown to preferentially affect these subtypes differently (Leggio et al., 2014).

Several studies have found that acute EtOH stimulates DA release in the NAc (Imperato & Di Chiara, 1986; Zapata et al., 2006). Moreover, there is also compelling evidence suggesting that EtOH can increase DA activity indirectly by acting on GABA neurons and opioid receptors in the NAc (Adermark et al., 2011). Here we found that Pb exposure only altered locomotor response to EtOH during day 3, the acute response. However, the control animals did not have an overall increased acute response to the first EtOH injection, as expected at a 2.0 g/kg dose (Crabbe & et al, 1982; Phillips et al., 1995; Rose et al., 2013). C57BL/6 mice typically show a modest increase in locomotor activity, which was seen in the first 5 minutes, but quickly fell below baseline activity as time went on. These results suggest that developmental Pb exposure altered DA neurotransmission and increased the stimulatory effects of EtOH in C57BL/6 mice, which is in line with the current literature given that this strain typically shows less DA release than DBA/2J mice when EtOH is systemically administered (Brodie et al., 1990; You et al., 2018). However, whether Pb alters DA release directly or indirectly cannot be elucidated based on these findings.

Currently, there are no studies that have looked at the direct effects of Pb and EtOH on DA activity. Thus, it would be important to probe the relationship between the two to improve our understanding of how Pb increases EtOH consumption but does not lead to EBS in this model. Specifically, it would be important to probe the effects of Pb on DA activity under conditions without a paired context, given that novelty was important for the increased locomotor response to acute EtOH in the Pb/CE group.

Further, Pb exposure alters various neurotransmission systems, such as GABA, NMDA, and DA activity. Thus, while an increase in DA receptors may have contributed to the initial acute response, the fact that Pb also disrupts GABA and NMDA neurotransmission cannot be ignored in the context of EBS. Specifically, here, since we did not see a sensitized response to EtOH. Next, very few studies have focused on the effects of DA receptors at induction and expression phases, and some studies have even found that DA activity is not as involved in EBS as other forms of drug-induced sensitization such as cocaine (Broadbent et al., 2005; Nashed et al., 2019; Zapata et al., 2006).

In experiment 3, Pb-exposed mice expressed a supersensitive response to repeated injections of 10 mg/kg cocaine, which suggests that Pb-exposed animals express a functionally hyperactive dopaminergic system. Previous experiments have shown that chronic Pb exposure in adulthood attenuates locomotor-stimulating properties of cocaine (Nation et al., 1996); however, in similar experiments, Pb exposure during developmental periods (gestation/lactation) increases locomotor activity after repeated systemic injections of cocaine (Nation et al., 2000). The findings in experiment 3 are in line with the current literature. Of note, however, is that the Pb levels in older experiments were high compared to what the current low levels are defined as.

Additionally, in adulthood, repeated exposure to cocaine has shown to decrease D1 and D2 receptors in the NAc (Dow-Edwards, 1989), which may explain why CBS persists long after the expression phase. Moreover, D2 knockout mice typically increase the rate at which they self-administer cocaine, while D1 knockouts decrease their self-administration behavior (Koob, Arends, & LeMoal, 2014). Further, Ma et al. (1999) found that Pb exposure decreased D2 binding affinity but found no differences in D1 binding affinity. Collectively, this information implies that D1 receptors may be driving the stimulatory properties of cocaine, and the decrease in D2 activity as a result of Pb exposure may be the reason animals are supersensitive to the stimulatory properties of the drug.

Next, Pb exposure during development facilitates D1 and D2 development in the striatum and NAc (Widzowsi et al., 1994). As a result, initial changes in D1 and D2 receptors may result in compensatory mechanisms, via changes in secondary messenger channels, that lead to increased expression of DA D1 receptors in later periods of life; however, there are no studies that have focused on alternative systems that could be mediating changes in the number of receptors or receptor binding affinity. This overall increase in receptors may contribute to why animals are supersensitive to the stimulating properties of cocaine.

Limitations and Future Directions

In agreement with previous research, Pb exposure increases voluntary EtOH consumption, increased EtOH sensitivity to an acute response, and increased sensitivity to the stimulatory properties of cocaine. It is evident that developmental Pb exposure at low levels may be altering the DA system in such a way that adults are more sensitive to the effects of drugs of abuse. However, there are several gaps that remain in the literature, as well as methodological

limitations and inconsistencies. Across studies, the species used, amount of Pb, method of Pb exposure, and length of time of exposure is variable. Thus, it is difficult to draw definite conclusions from the few studies looking at the effects of Pb on drug response. For example, most studies have used rats instead of mice, which were used here. In these experiments, C57BL/6 mice were used to assess behaviors; however, there are no other studies using this strain to understand Pb exposure in animal models, making it difficult to compare findings directly.

Additionally, the amount of exposure and the developmental vs. real-time effects is important to consider. While here we used a model that has shown to yield BLLs < 10 μg/dL in pilot studies (unpublished), previous studies have used much higher levels. Given that Pb levels have dropped to yield BLLs much lower than what was seen earlier, this study provides more translational findings and corroborates the work of seminal human studies, which found that children were more vulnerable to the effects of Pb than adults (Dietrich et al., 1993; Shukla et al., 1989). Further, among preclinical models, many studies exposed animals prenatally or began exposure at prenatal periods and continued for a few weeks postnatally. This kind of exposure allows for different questions to be asked due to evidence suggesting the amount and length of exposure period change behavior in different manners. Further, while it is assumed that there may not be any detectable levels of Pb in the adult mice used here, we cannot be confident in this statement as Pb can settle in bones and other hard tissues slowly allowing Pb to reenter the bloodstream. Thus, while studies have focused on the effects of Pb exposure on different life stages, it is difficult to disentangle and firmly attribute underlying mechanisms to behavioral changes because they could be due to biochemical disruptions during development/maturation or disruptions that are a result of real-time Pb effects, which future studies should consider.

Lastly, in these experiments, there were a few limitations. While we can confidently say that our model altered DA-dependent behaviors and drug consumption, no blood samples were collected and analyzed to confirm Pb levels nor EtOH levels in experiment 2. As mentioned previously, our methods were adapted from Sobin et al. (2009), which has consistently yielded BLLs translational to what is seen in children living in low socioeconomic environments today (Sobin et al., 2009, 2013; Tena et al., 2019). Future experiments will focus on assessing BLLs, bone Pb levels, and brain Pb levels at PND 42, immediately after the Pb exposure period, and at PND 60+ to confirm Pb levels in our mice before and at the time of testing. Next, while both

sexes were included, sex effects were not initially hypothesized. Some studies have alluded to there being sex-specific differences in behaviors following Pb exposure in both preclinical and clinical studies (Singh et al., 2018; Sobolewski et al., 2018) in behaviors such as anxiety and learning and cognition tasks. Here we did not find any beyond EPM latency time in the EPM test. Moving forward, studies should focus on sex-specific differences using a similar paradigm in order to understand if low levels affect drug sensitivity differently in males and females.

Conclusion

The accumulated data assessing early-life, low-level Pb exposure, translational to children living in urban and/or low socioeconomic areas in the United States today, reveals that DA-dependent behaviors in rodent models are altered. This is the first experiment to use a consistent Pb-exposure protocol to assess if early Pb exposure can alter a variety of behaviors associated with addiction in adulthood. While there were no major differences in anxiety-like behaviors, acute responses to EtOH and overall responses to cocaine were significant. This enhanced sensitivity to these two drugs of abuse is evidence that children living in areas with higher concentrations of Pb are at risk for potential substance use disorders and even more at risk if the first initial use is during adolescence (Nation et al., 2000). While the underlying neurochemical mechanisms are not fully understood, these studies provide concrete evidence that Pb increases drug sensitivity. Thus, more intervention and resources are needed in areas where children are exposed to higher concentrations of Pb.

FIGURES AND TABLES

Table 1. The number of animals per group organized by experiment.

Table 1 The number of animals in each group by experiment

	Pb Male	Pb Female	No Pb Male	No Pb Female	Pb Male	Pb Female	No Pb Male	No Pb Female				
Experiment	**3W**	**3W**	**3W**	**3W**	**0W**	**0W**	**0W**	**0W**				
1	7	7	7	7	7	7	7	7				
	Pb Male	Pb Female	No Pb Male	No Pb Female	Pb Male	Pb Female	No Pb Male	No Pb Female	Pb Male	Pb Female	No Pb Male	No Pb Female
	CE	**CE**	**CE**	**CE**	**CS**	**CS**	**CS**	**CS**	**SC**	**SC**	**SC**	**SC**
2	10	6	9	7	11	5	9	8	9	6	9	7
	Pb Male	Pb Female	No Pb Male	No Pb Female	Pb Male	Pb Female	No Pb Male	No Pb Female	Pb Male	Pb Female	No Pb Male	No Pb Female
	CC	**CC**	**CC**	**CC**	**AC**	**AC**	**AC**	**AC**	**SC**	**SC**	**SC**	**SC**
3	5	5	5	5	5	5	6	6	5	5	5	4

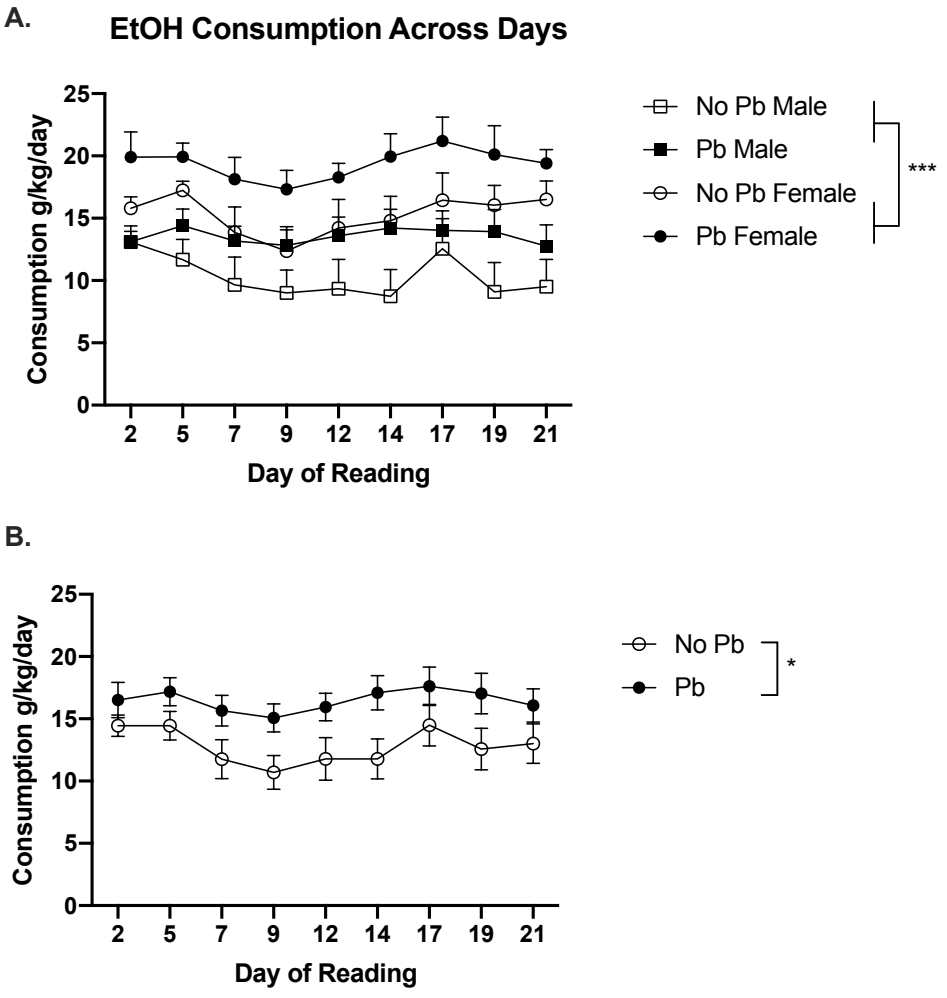


Figure 1. Two-bottle choice EtOH Consumption.

Developmental Pb exposure increases voluntary EtOH consumption in a two-bottle choice paradigm in both male and female C57BL/6 mice. A) Females consumed higher volumes of EtOH than males. B) Pb-exposed animals consumed more EtOH than control animals, regardless of sex. (*) $p < 0.05$, (***) $p < 0.001$

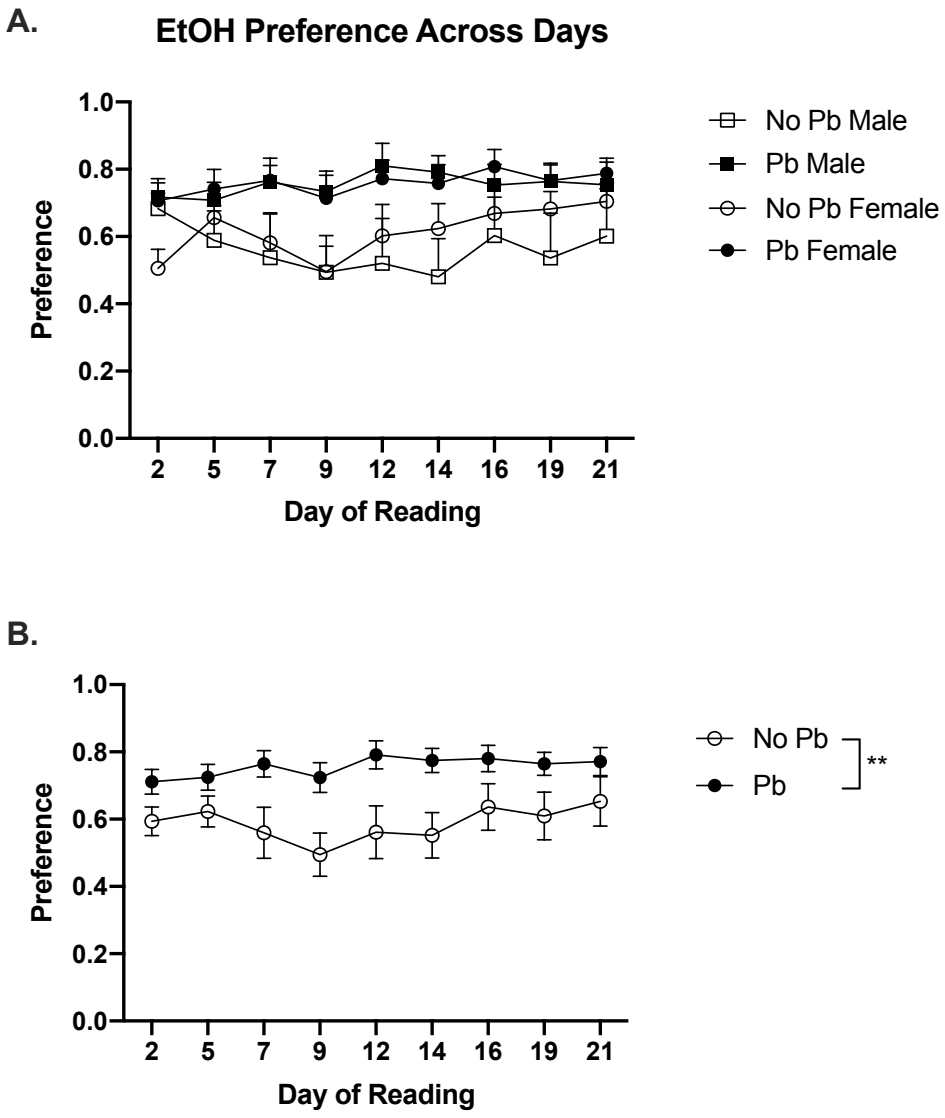


Figure 2. Two-bottle choice EtOH preference.

Developmental Pb exposure increases the overall preference for EtOH in both male and female C57BL/6 mice. A) There is no effect of sex on preference drinking; however, when collapsed, B) Pb-exposed animals have a higher preference for EtOH than animals in the control group. (**) $p < 0.005$

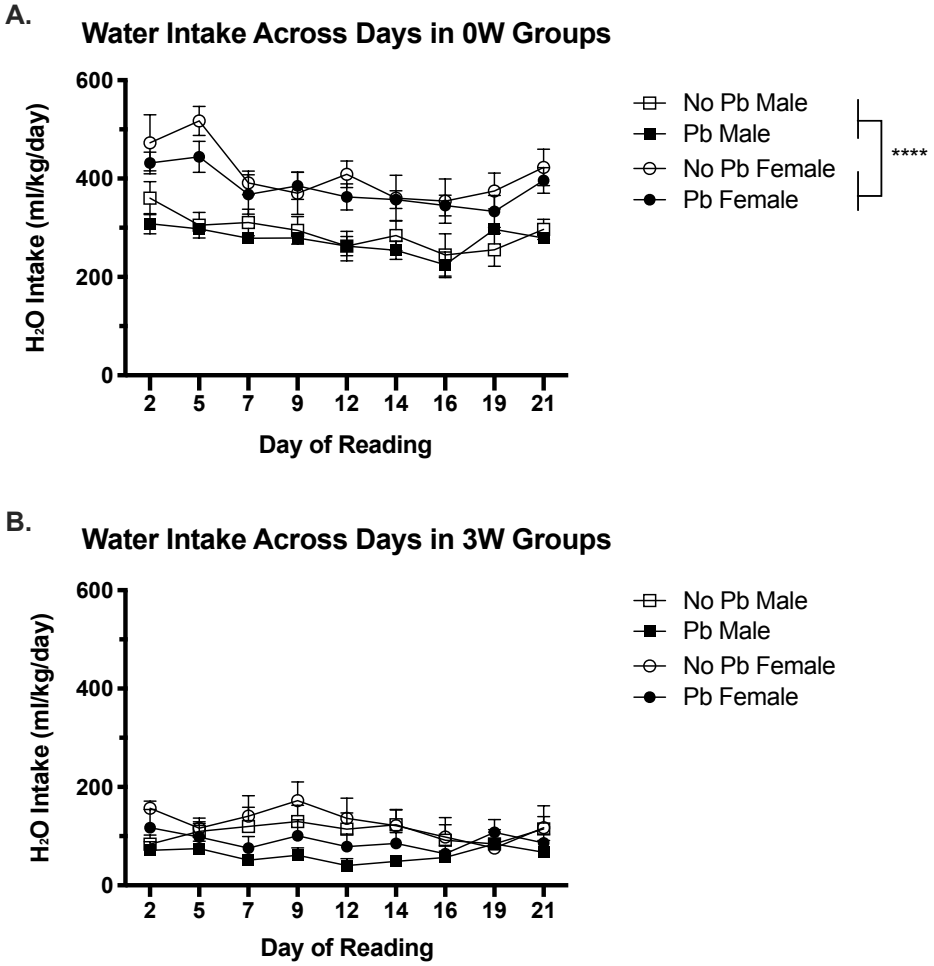
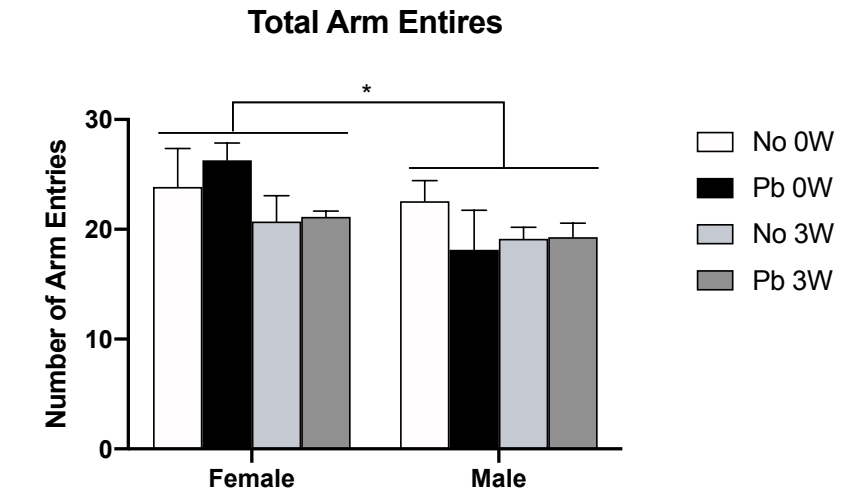


Figure 3. Developmental Pb exposure does not change mean water intake.

A) While there is no difference in total fluid intake in the 0W animals between Pb and no Pb control groups, females consistently drink more than males across days. B) There were no differences in water intake across days between Pb and No Pb groups; however, there was an interaction between Day and Pb exposure. (****) $p < 0.0001$

A.



B.

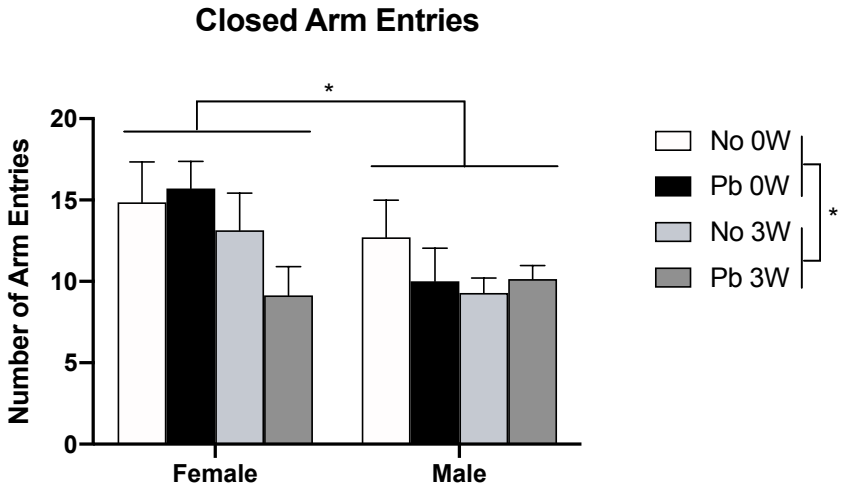


Figure 4. Crude locomotor activity.

A) Females exhibited higher locomotor activity as defined by more total arm entries than males. B) Females entered the open arm entries more than males, suggesting less anxiety-like behavior than males. Groups with a history of EtOH exhibited anxiolytic behavior as compared to the water control groups, regardless of Pb exposure.
(*) $p < 0.05$

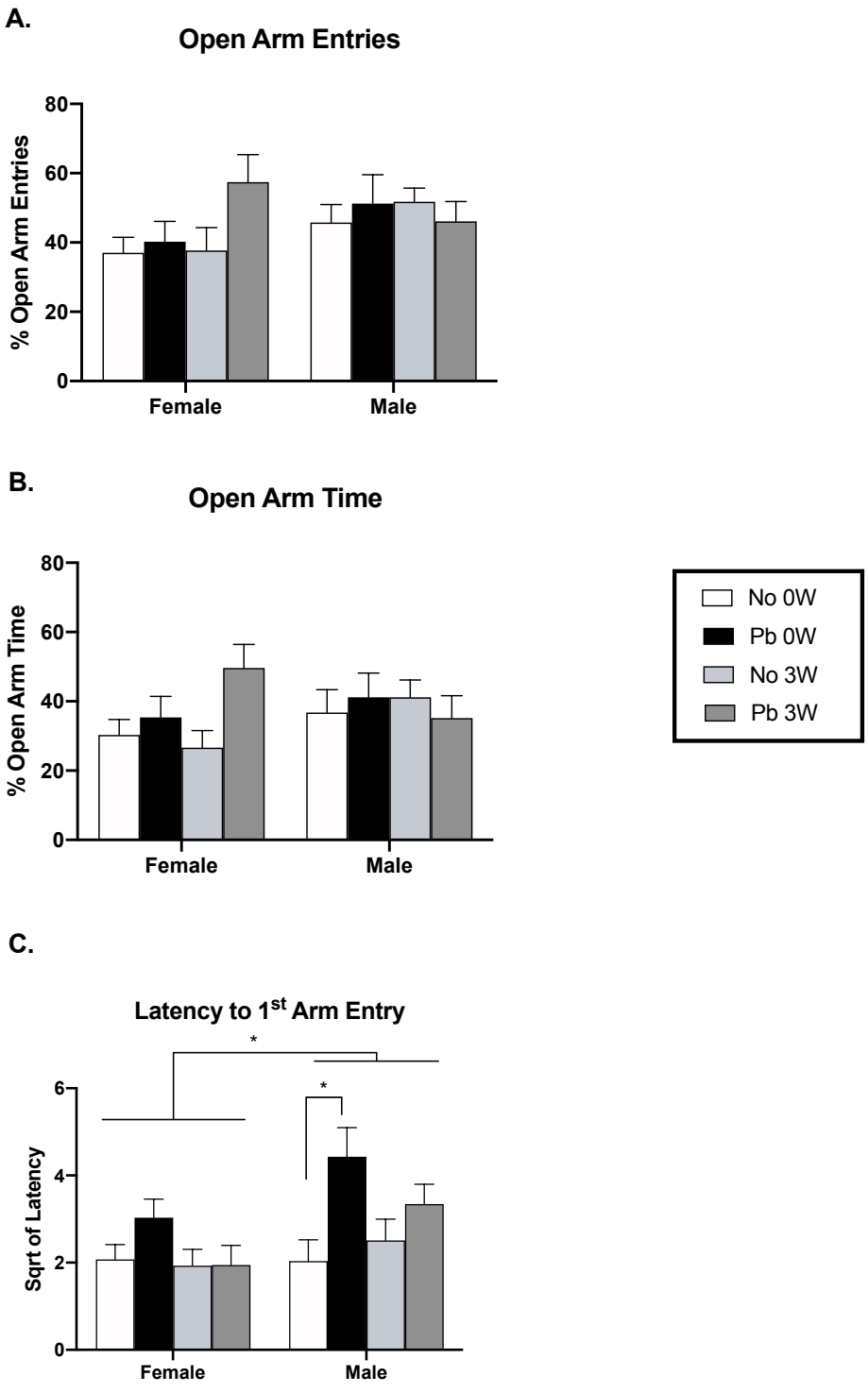


Figure 5. There were no differences in anxiety-like behavior as measured by the EPM.

A) % open arm entries and B) % open arm time. C) There was a difference in latency to enter the first arm with males deliberating longer as well as Pb exposed animals compared to any other group. (*) $p < 0.05$

Table 2. Ethanol-induced locomotor sensitization paradigm

Table 2 Ethanol-induced locomotor sensitization paradigm

Treatment groups	Open Field Day 0	Habituation Days 1-2	Acute EtOH Day 3	Daily treatment (Induction phase) Days 4-13	Sensitization test (Expression phase) Day 14
Chronic EtOH (CE)	No injection	Saline	EtOH (2 g/kg)	EtOH (2.5 g/kg)	EtOH (2 g/kg)
Chronic Saline (CS)	No injection	Saline	Saline	Saline	EtOH (2 g/kg)
Saline Control (SC)	No injection	Saline	Saline	Saline	Saline

EtOH Ethanol

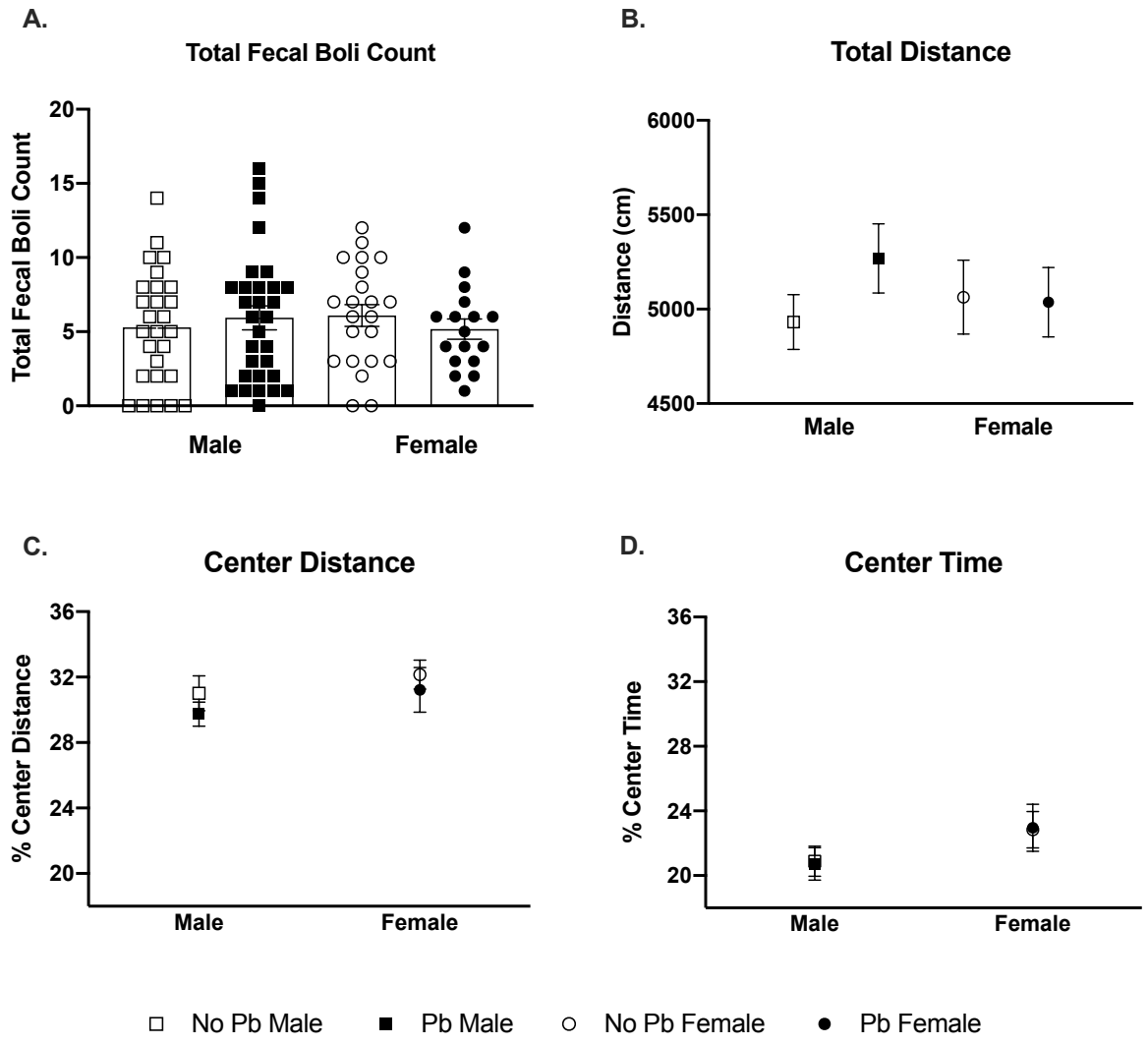


Figure 6. Open Field Test.

Pb-exposed mice did not exhibit anxiety-like behaviors compared to controls as measured though A) fecal boli count, B) total distance, C) % center distance, and D) % time spent in the center.

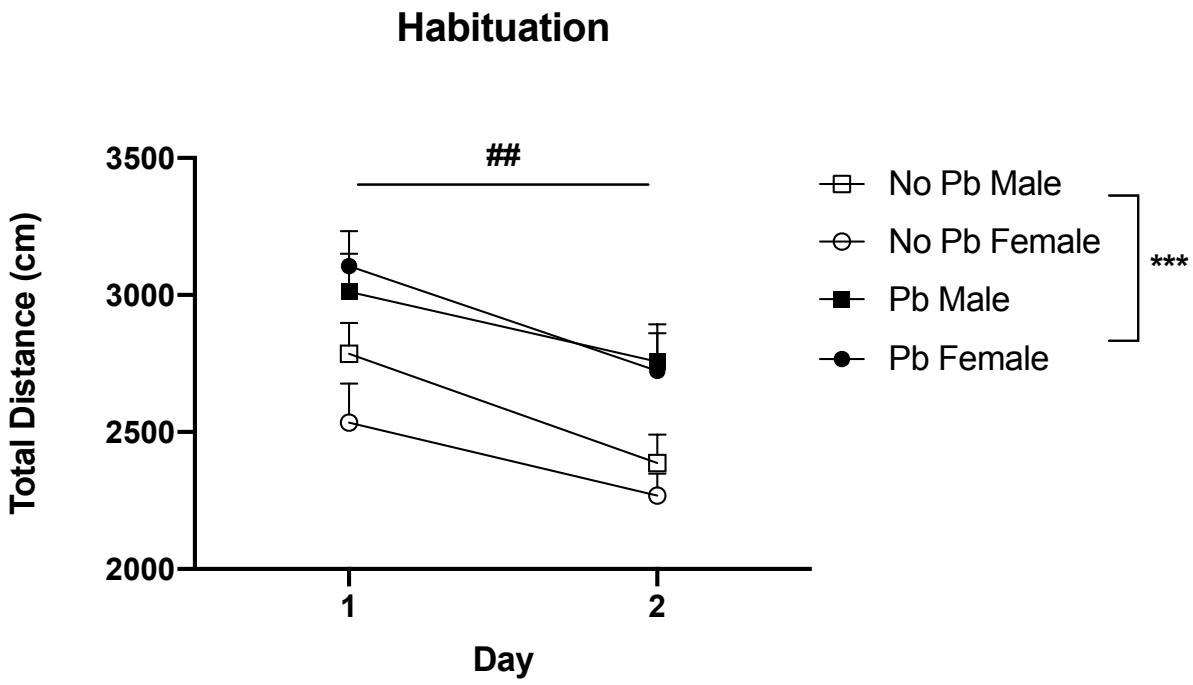


Figure 7. Locomotor activity (total distance) during habituation days 1 and 2.

Pb-exposed animals exhibited enhanced locomotor activity as compared to controls. (***) $p < 0.001$, (##) between days $p < 0.001$

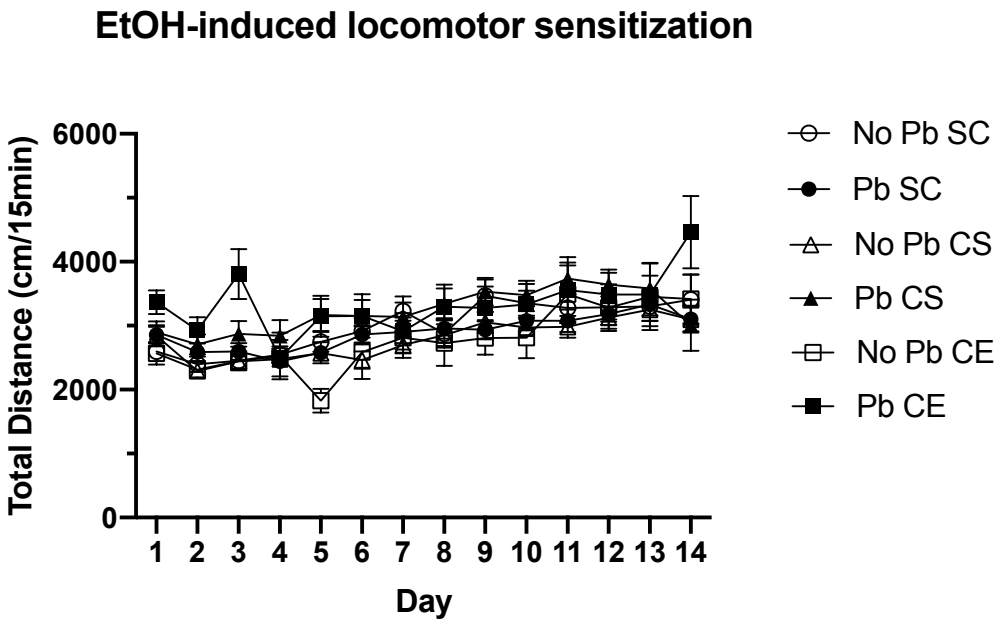
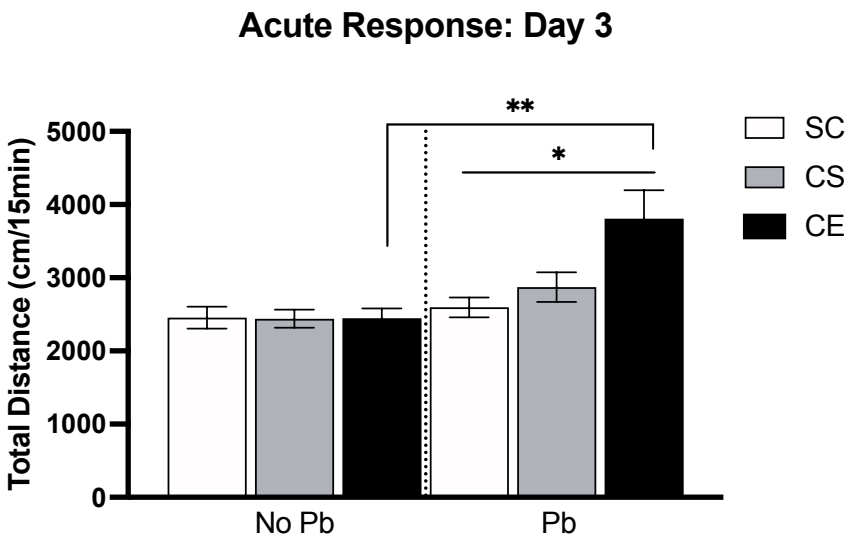


Figure 8. EtOH-induced locomotor sensitization across days broken down into groups. ***CE****= chronic ethanol,* ***CS****= chronic saline, and* ***SC****= saline control*

A.



B.

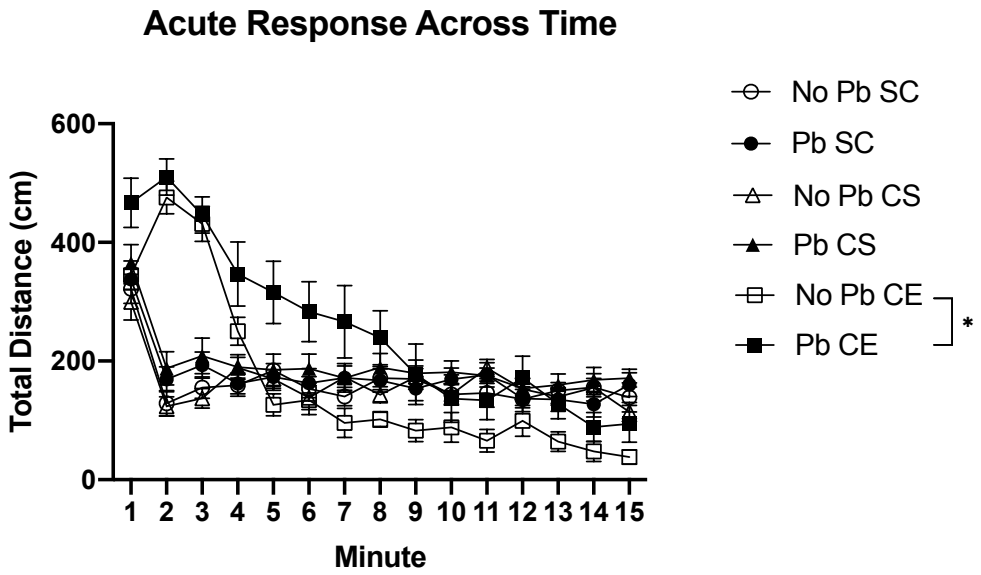


Figure 9. Locomotor activity level on day 3 acute response to 2.0 g/kg EtOH.

A) Pb/CE group showed an increase in activity compared to the CS and SC groups on day 3. The Pb exposed group was also more sensitive to the stimulatory properties of EtOH on day 3 than control animals in the CE group. B) Pb exposed animals in the CE group had increased locomotor activity as compared to the No Pb/CE group and the Pb/SC group. (*) $p < 0.05$, (**) $p < 0.01$

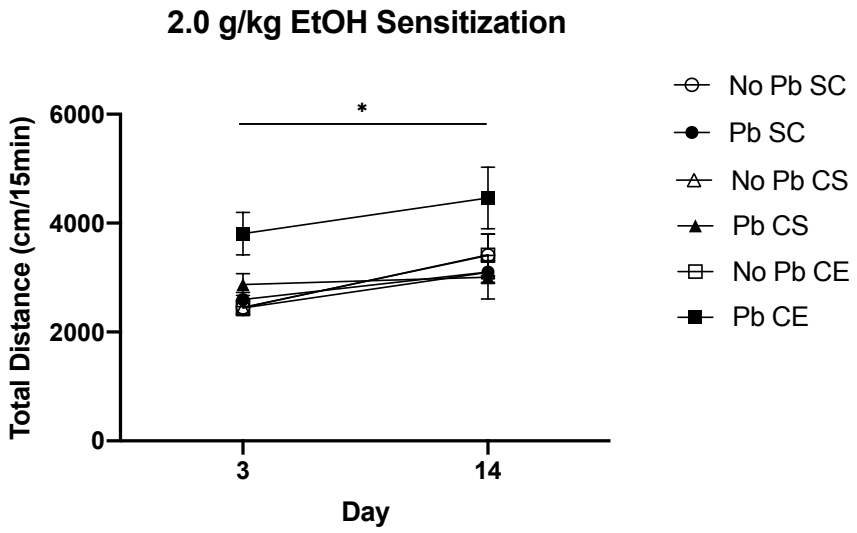


Figure 10. Within-group sensitization.

The No Pb/CE group sensitized to 2.0 g/kg EtOH, but not the Pb/CE group. (*) $p < 0.05$

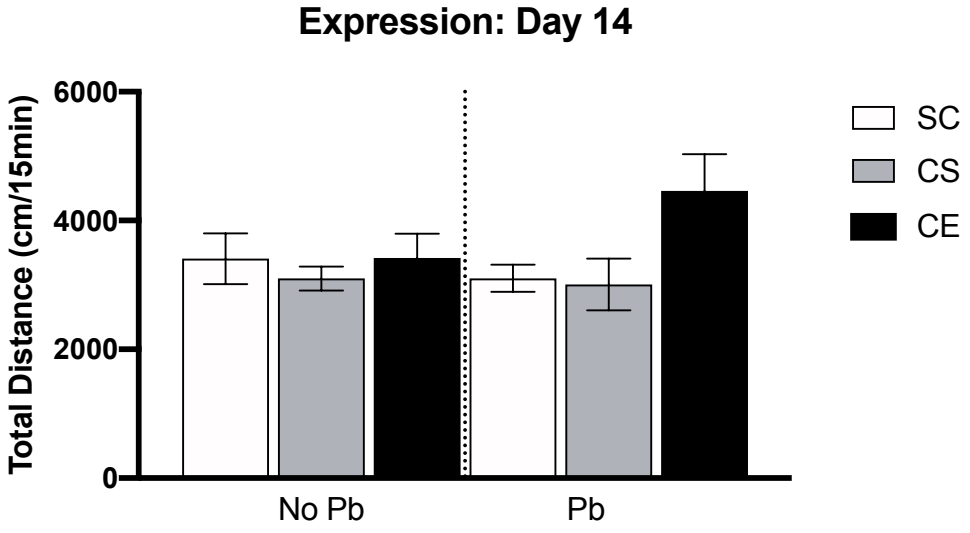


Figure 11. Expression Day.

On expression day, there were no significant difference between any of the groups ($p > 0.05$).

Table 3. Cocaine-induced locomotor sensitization paradigm

Table 3 Cocaine-induced locomotor sensitization paradigm

Treatment groups	Habituation Days 1-2	Acute EtOH Day 3	Daily treatment (Induction phase) Days 5, 7, 9	Sensitization test (Expression phase) Day 11	Challenge Day 19
Chronic Cocaine (CC)	Saline	Cocaine (10 mg/kg)	Cocaine (10 mg/kg)	Cocaine (10 mg/kg)	Cocaine (10 mg/kg)
Acute Cocaine (AC)	Saline	Saline	Saline	Cocaine (10 mg/kg)	Cocaine (10 mg/kg)
Saline Control (SAL)	Saline	Saline	Saline	Saline	Saline

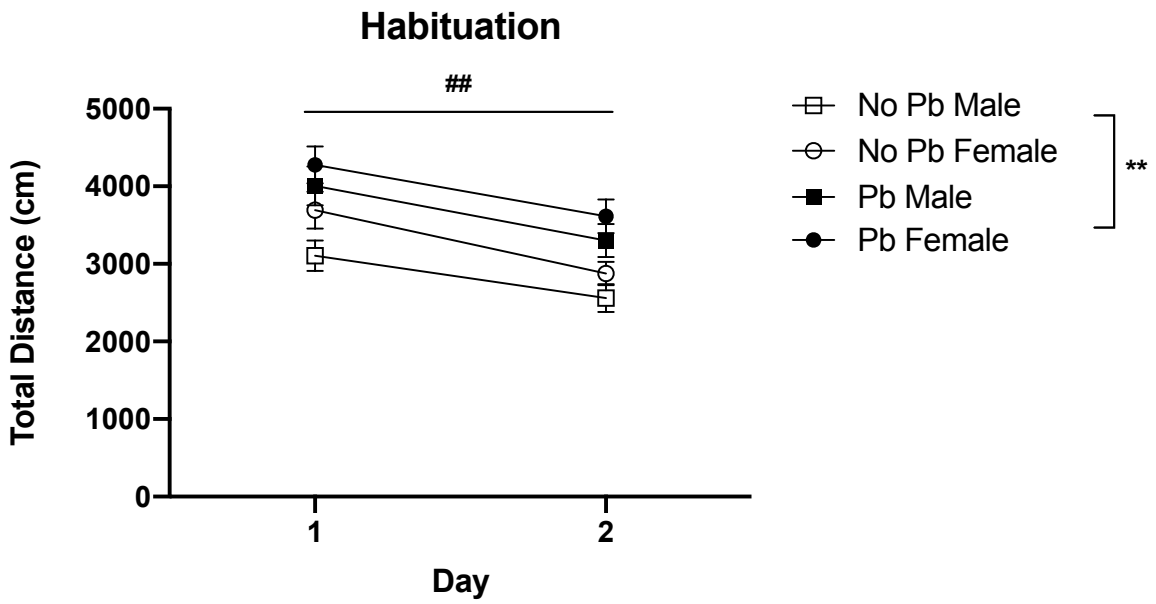


Figure 12. Locomotor activity (total distance) during habituation days 1 and 2.

Pb-exposed mice exhibited heightened locomotor activity compared to controls. (**) $p < 0.01$, (##) between days $p < 0.001$

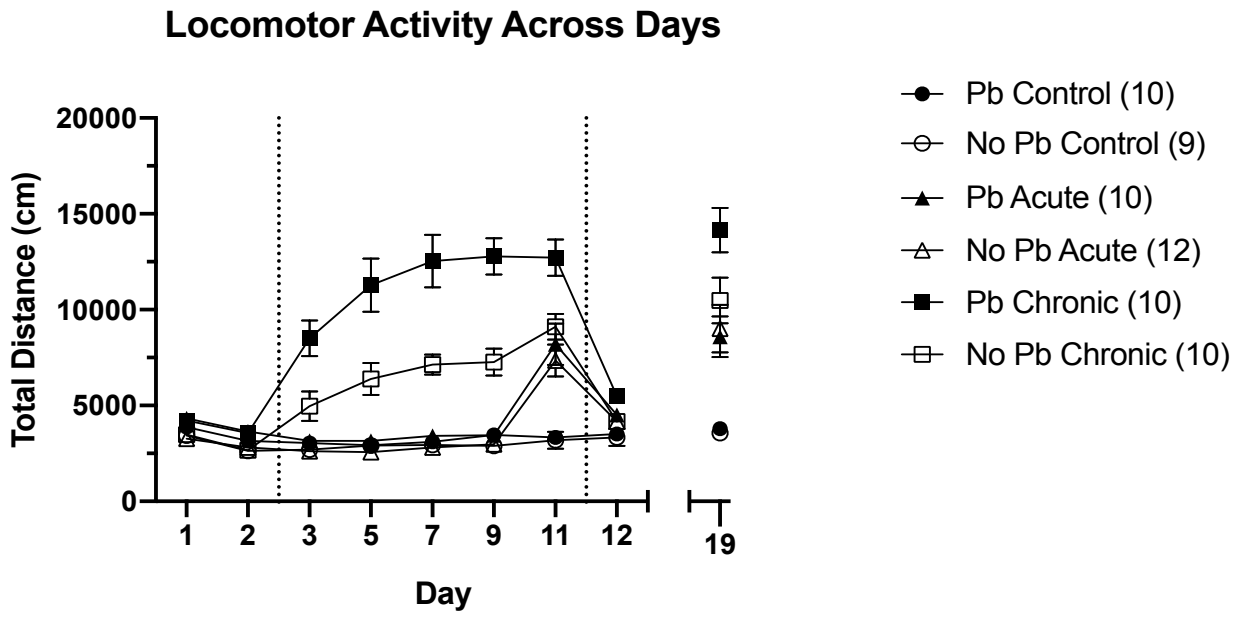


Figure 13. Complete sensitization protocol across days collapsed by sex.

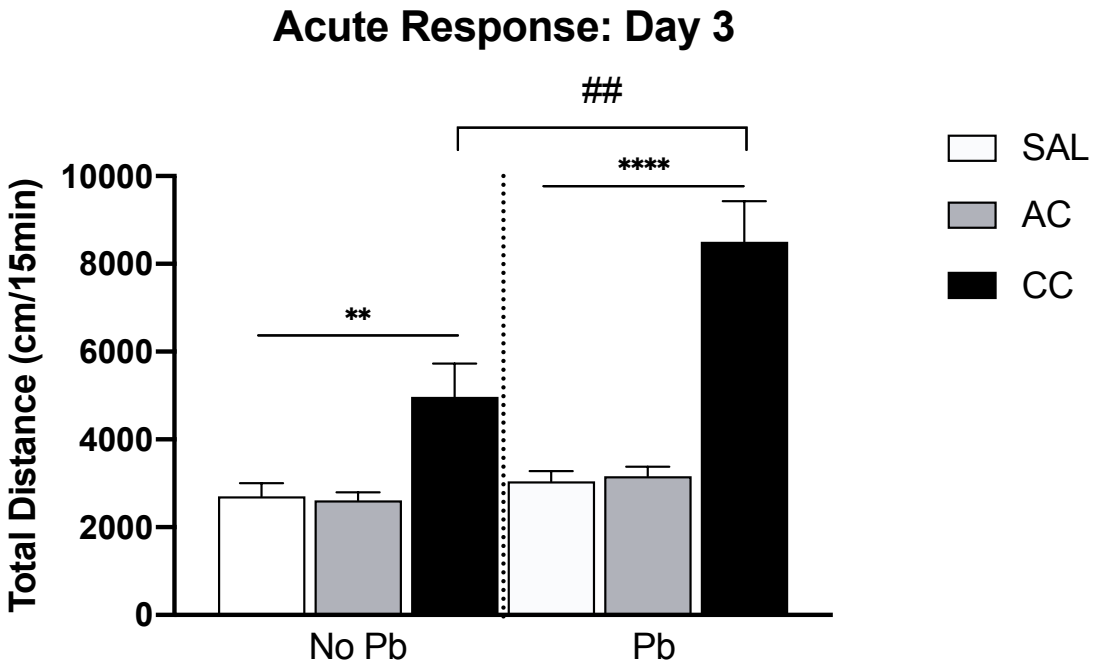


Figure 14. Acute response to 10 mg/kg cocaine in Pb and No Pb groups.

In the chronic groups (CC), Pb-exposed mice were more sensitive to the stimulatory effects of cocaine than the No Pb/CC group. Line indicates CC vs AC and SAL (**) $p< 0.01$, (****) $p< 0.0001$, (##) between exposure groups $p< 0.01$

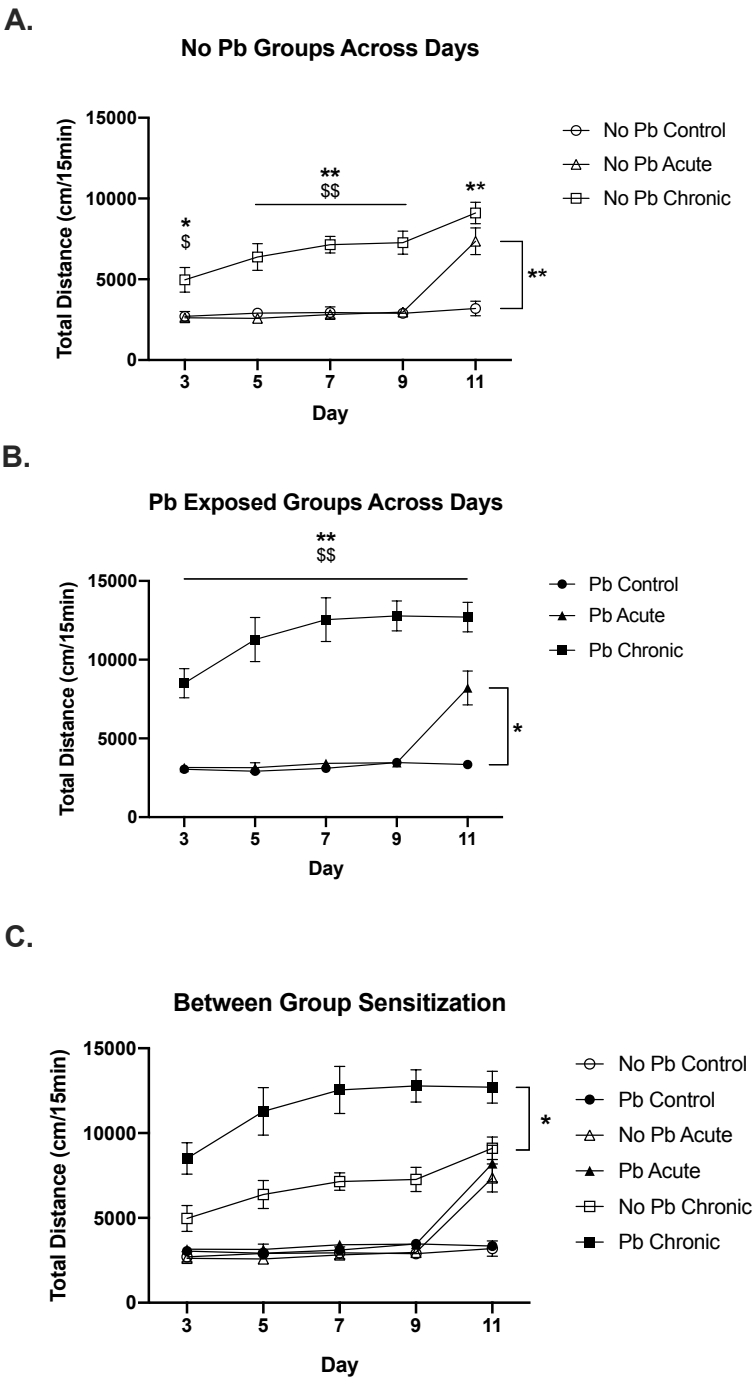


Figure 15. Locomotor activity of each group across induction (3-9) and expression phase (11).

Groups were collapsed and analyzed by A) No Pb groups and B) Pb groups. C) shows between-group differences. Between CC and SC (*) $p< 0.05$, (**) $p< 0.01$, (***) $p< 0.001$, (****) $p< 0.0001$; between CC and AC ($) $p< 0.05$, ($$) $p< 0.01$

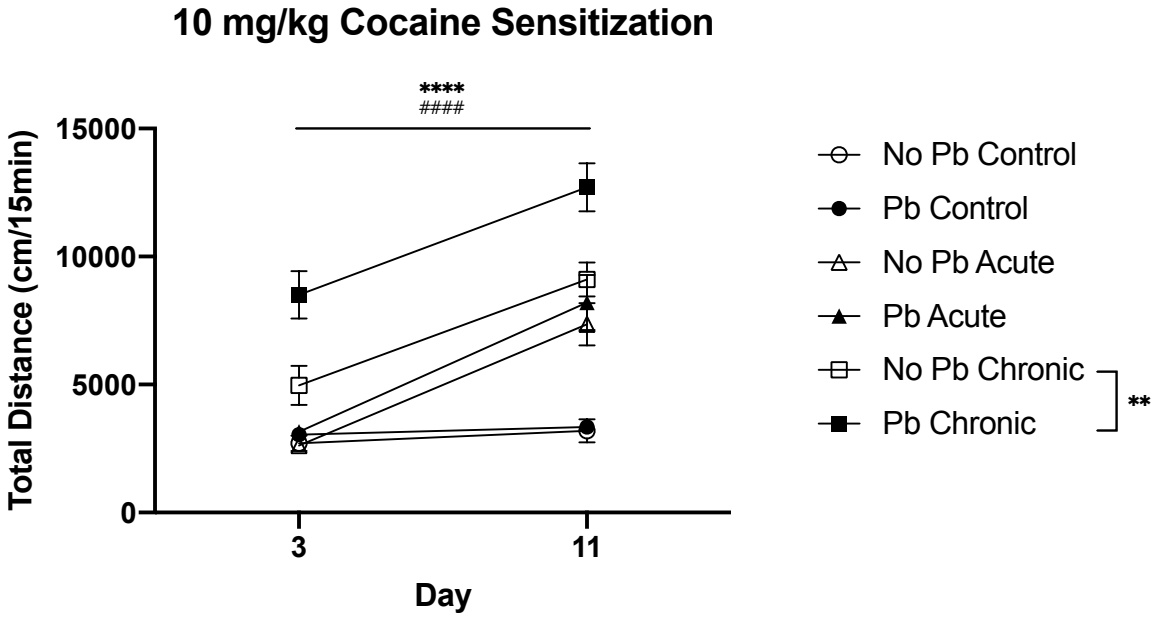


Figure 16. Within-group sensitization.

Both the Pb/CC and the No Pb/CC groups sensitized to 10 mg/kg cocaine and the Pb-exposed animals showed a supersensitive response compared to the No Pb controls. Pb sensitization (****) $p < 0.0001$; No Pb sensitization (####) $p < 0.0001$; Between CC groups (**) $p < 0.01$.

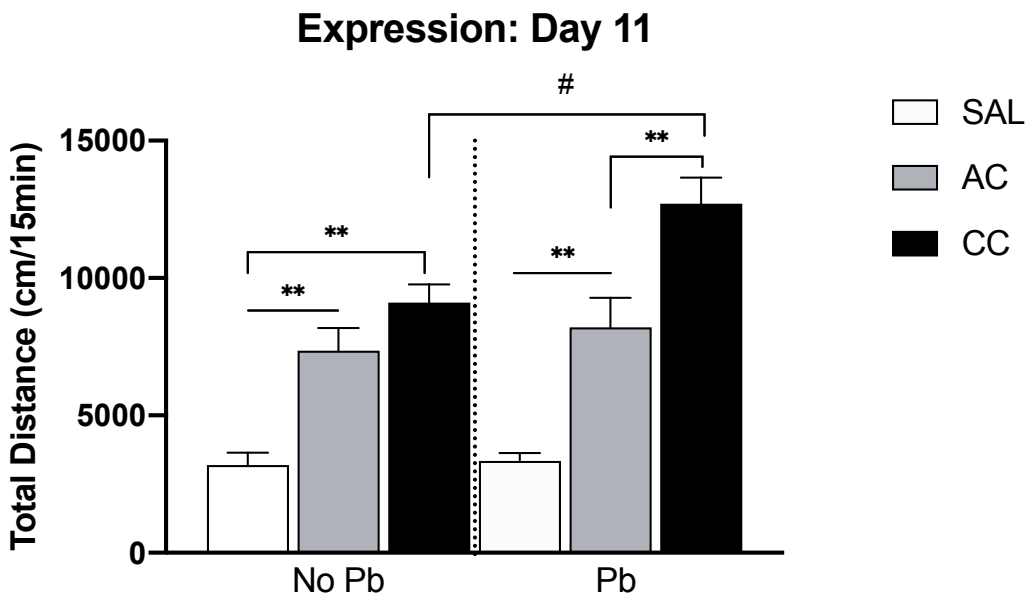


Figure 17. Expression phase (day 11) of sensitization protocol.

Pb-exposed animals in CC group exhibited increased locomotor response compared to No Pb/CC group, Pb/AC group, and Pb/SC group. (**) $p < 0.01$, (***) $p < 0.001$, (#) between groups $p < 0.05$

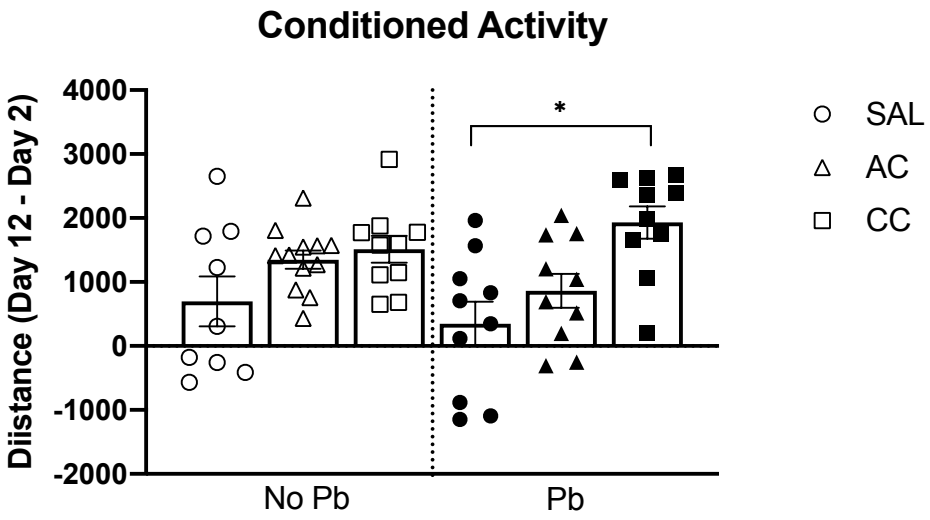


Figure 18. Conditioned locomotor activity displayed as day 12 – day 2.
(*) $p < 0.05$

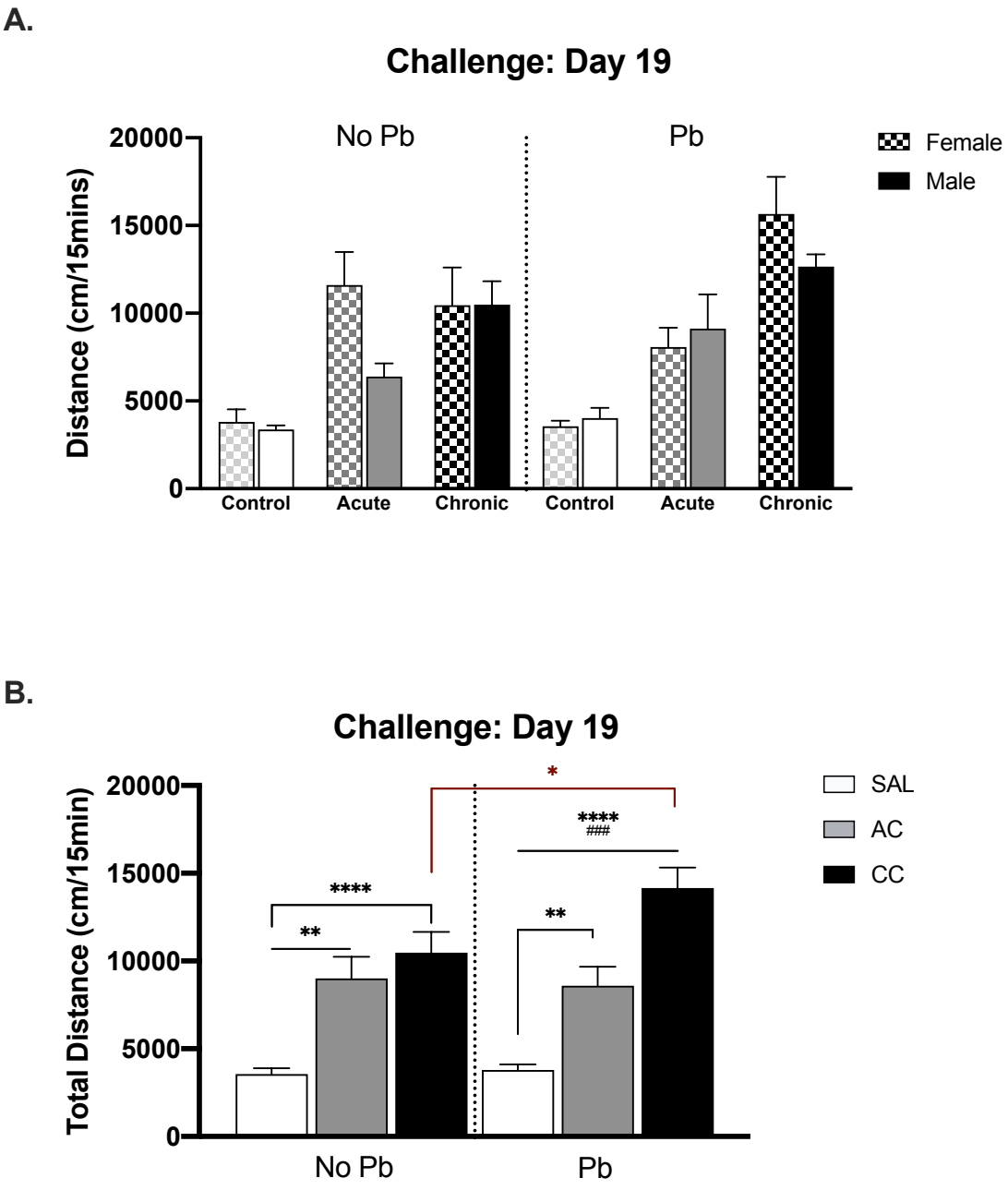


Figure 19. Locomotor activity on day 19 (challenge day).

While there was no main effect of sex, there was a trend (p=0.056), so A) groups are broken down by males and females. Groups were collapsed and B) shows between-group differences. The Pb/CC continued to show a heightened locomotor response to 10 mg/kg compared to the No Pb/CC group following a one-week withdrawal period. Red (*) p< 0.05; between groups (**) p< 0.01, (****) p< 0.0001, Pb/CC vs Pb/AC (###) p< 0.001

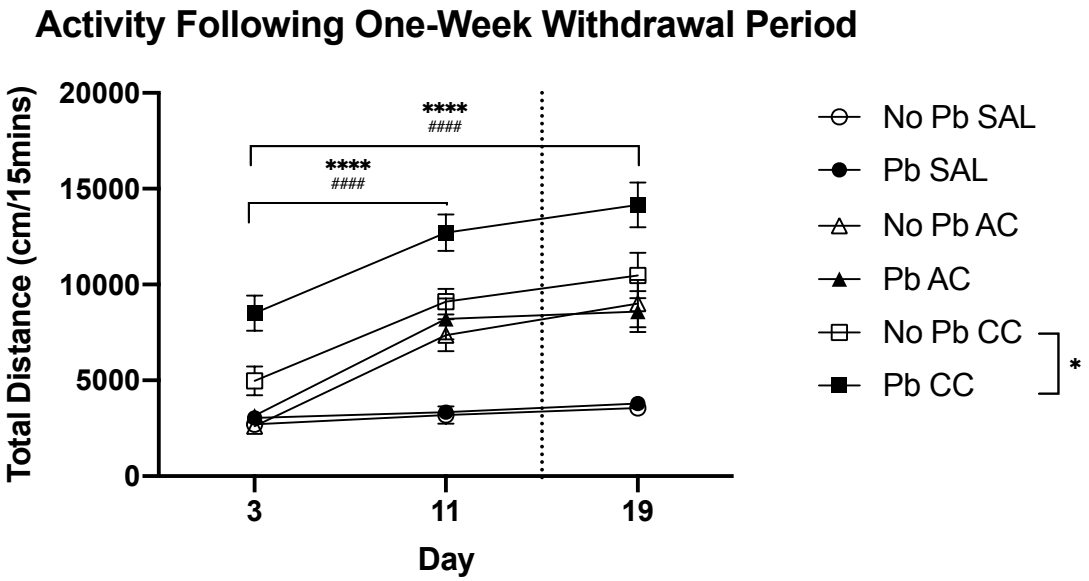


Figure 20. Activity across acute, expression, and challenge days.

Pb-exposed animals in the chronic group exhibited heightened locomotor activity compared to controls in the chronic group across days. No group showed significantly different activity on day 19 compared to day 11; however, there was a significant difference between day 19 and day 3 in both the Pb/CC and No Pb/CC groups ($p < 0.0001$). No Pb/CC (####) $p < 0.0001$), Pb/CC (****) $p < 0.0001$

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