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A Mouse Model Of Toluene Abstinence-Induced Pathology

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A MOUSE MODEL OF TOLUENE ABSTINENCE-INDUCED PATHOLOGY

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DEDICATION

To my wife Jade for putting up with all my insanity.

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CHAPTER 1: INTRODUCTION

The deliberate inhalation of organic solvents, such as toluene, continues to be a persistent public health issue with 6.3% of teens reporting that inhalants were the first class of drugs they abused (SAMHSA, 2014). Despite the high risk of serious harm or death (Butland, Field-Smith, Ramsey, & Anderson, 2012), inhalants remain one of the most commonly abused drug types in developing countries (Howard, Bowen, Garland, Perron, & Vaughn, 2011). In the United States, inhalant abuse is particularly prevalent among younger individuals, with 14.9% of eighth grade students admitting to having misused inhalants (SAMHSA, 2012). While inhalant abuse often begins in adolescence, a number of individuals that start early actually continue to misuse inhalants into adulthood (SAMHSA, 2014). The misuse of inhalants is life threatening with numerous reported fatalities resulting from accidental overdose (Beasley, Frampton, & Fountain, 2006; Bowen, 2011; Bowen, Daniel, & Balster, 1999; Field-Smith, 2002; Maxwell, 2001; Wick, Gilbert, Felgate, & Byard, 2007). Clinical evidence indicates that individuals develop longstanding patterns of recurring inhalant abuse (Verma, Balhara, & Dhawan, 2011) and experience difficulty maintaining sobriety. Additionally, individuals who attempt inhalant sobriety experience negative symptoms that abate when the individual relapses and begins reusing the inhalant (Shah, Vankar, & Upadhyaya, 1999). This is true for toluene, one of the most commonly abused inhalants (A. C. Evans & Raistrick, 1987) 1987). Indeed, toluene abusers report symptoms of anxiety, aggression, and even peripheral tremors beginning within days of ceasing toluene use (Kouzoupis, Konstantakopoulos, Oulis, Kalfakis, & Papageorgiou, 2010). Despite the clinical documentation of an abstinence syndrome, there are no published reports of the behavioral effects of toluene abstinence using an animal model. This is of critical importance because the adverse symptoms that arise during abstinence / withdrawal may predispose individuals to relapse. There has been a

tendency in the inhalant abuse literature to focus on inhalants' positive reinforcing effects (e.g., studies of central dopamine dynamics, self-administration, or drug discrimination). However, the motivating effects that occur during withdrawal are equally important. Negative affective states are known to precipitate relapse (Koob, 2009 for a review). This is troubling as relapse rates for most addictive drugs are high (Darker, Sweeney, Barry, Farrell, & Donnelly-Swift, 2015; Schoenthaler et al., 2015), suggesting that the majority of drug abusers face at least initial difficulty in maintaining abstinence. The available evidence suggests that this pattern holds true for inhalant abuse as well (Akoijam, Jamir, Phesao, & Senjam, 2013). Awareness of withdrawal signs becomes a critical component in effectively treating a substance abuse disorder (e.g., Thurgood, McNeill, Clark-Carter, & Brose, 2016). As such, it is critical to better understand inhalant withdrawal to gain correct perspective on effectively treating individuals who habitually misuse or are dependent upon products containing inhalants such as toluene.

At present, information regarding the symptoms that arise during abstinence from toluene abuse is limited to descriptive clinical studies (see Inhalant Withdrawal, pg. 11). Indeed, withdrawal remains a chronically understudied facet of toluene abuse, despite evidence suggesting that withdrawal symptoms are both serious and likely negative reinforcers of inhalant abuse in humans. Principally, it is important to the development of the inhalant abuse research field to investigate toluene withdrawal in an animal model, and there is a robust body of alcohol (ethanol) withdrawal literature (See pharmacodynamic properties of toluene, pg. 8) to utilize as a guide for developing testable hypotheses. Ethanol (ETOH) and toluene share many pharmacodynamic properties, making ETOH a logical starting point for developing methods of examining toluene withdrawal. However, toluene and ethanol differ in potency and route of administration and as

such we cannot simply extrapolate findings from the ETOH literature – direct experimental examination of toluene withdrawal is necessary.

The goal of the present research was to expand our understanding of toluene abstinence using well-established solvent exposure models in mice. The contribution of this research project is that the behavioral effects of toluene abstinence in mice were characterized. This contribution is important because it allows for a better understanding of factors that predispose individuals towards toluene relapse.

1.1: Relevant Terminology

Terms used here alternate between "inhalants" or "solvents" and the more specific compound "toluene." Because toluene is so commonly studied compared to other inhalants, and the direct biobehavioral effects of toluene exposure are relatively well understood, toluene is a logical initial choice for examining inhalant withdrawal. While the focus of this project was toluene abuse specifically, a longstanding issue with research on human inhalant abuse is a poor recognition that the *specific* inhalant an individual is using/misusing. This distinction is important because of differences in their effects, tolerance and withdrawal limit generalizability from any one, including toluene. Most national surveys (e.g., SAMHSA, 2014) of drug abuse and many human clinical studies of inhalant abuse do not adequately delineate among classes and types of inhalants. While the present research is widely relevant because it focuses on one of the most commonly misused inhalants, and may be a "best approximation" of "inhalants" as a broad class, generalizability will be limited. This dissertation uses the specific term "toluene" where possible, but where such specificity is not available, the more general terms "inhalants" or "solvents" will be used.

Additionally, it is important to distinguish among withdrawal, abstinence and dependence. Dependence is defined classically as the perceived need, driven by physical and/or psychological factors, to continue using a drug to maintain daily function, typically brought on by habitual exposure to that drug. In dependence, the absence of the drug produces physiological or psychological reactions identified, respectively, by "withdrawal" signs and craving. Withdrawal is therefore operationally defined as a state of drug absence following drug intoxication, while "withdrawal syndrome" refers to the constellation of signs/symptoms that arise in reaction/response to the user entering drug withdrawal. "Abstinence," while often used interchangeably with withdrawal, operationally refers to a period of drug absence, and any physiological changes that absence might precipitate, without presupposing physical dependence. As such, "drug abstinence" is a term that can be used in the study of withdrawal phenomenon prior to the confirmation of a "withdrawal syndrome." The concept of "craving" refers to the psychological state that motivates drug seeking behavior. Abstractly, it is the adverse sensation of desire for drug paired with perseverative thinking about drug. In operant conditioning experiments with animals, craving is inferred from the state of abstinence in which an animal is motivated to work for drug reward. As such, "craving" can be thought of as the motivational drive component of a "withdrawal syndrome."

1.2: Toluene

A considerable part of the problem with studying inhalant abuse is the heterogeneity of the inhalant class. The term "inhalants" is best thought of as an umbrella term referring to a shared method of administration of solvent vapors rather than shared chemical, pharmacokinetic or pharmacodynamics properties. In this view, an "inhalant" refers to a misused substance that is administered via inhalation that is *not* already classified in another category (e.g., tobacco,

crack/cocaine, methamphetamine, marijuana, etc.). In basic processes studies of inhalants, the umbrella term is more generally divided into sub-categories by chemical structure and function (e.g., organic solvents such as toluene, versus volatile anesthetics like nitrous oxide). However, all inhalants share some abuse potential and are potentially dangerous to the health of the user. The common method of administration is straightforward involving the user breathing deeply from a closed container or object saturated in some amount of the substance (Bowen, 2011). In general, the intoxicating effects of such a method persist for upwards of an hour (Bowen, 2011), though duration can vary depending on the substance used.

Toluene (methylbenzene) is an organic solvent with a high rate of abuse in many countries (Cruz, 2011). Indeed, solvents like toluene are amongst the most readily available and frequently misused inhalants worldwide (Bowen & Cruz, 2014for a review). Products containing toluene include airplane glue, some forms of gasoline, paint thinner, spray paint, and sealants (Hass, Lund, Hougaard, & Simonsen, 1999). Industrial and household products containing some amount of toluene are produced in excess of millions of tons per year (Agency for Toxic Substances and Disease RegistryATSDR, 2000). These products are often cheap and legally obtained by anyone. This makes toluene an especially easy compound to acquire and misuse/abuse.

Toluene displays many properties in common with other abused substances, including ETOH. The habit-forming potential of inhalants can be observed in the human literature, as 79.1% of Asian Indian schoolchildren who have experimented with inhalants have tried to stop unsuccessfully at least once, and over 50% of those who had experimented had abused inhalants the past 30 days (Akoijam et al., 2013). These results are consistent with a prior report from an Indian substance abuse clinic, where 57.1% of adult inhalant abusers surveyed had relapsed to continued abuse (Kumar et al., 2008). Evidence suggests that as many as one in five American

inhalant users eventually progress to meeting criterion for abuse, or an inhalant use disorder (Perron, Howard, Vaughn, & Jarman, 2009), suggesting that inhalants possess significant risk of dependence. The DSM-5 diagnosis "Inhalant Use Disorder" (304.60) uses criteria similar to many other substance use disorders. Increased use, use at inappropriate times, failure to reduce use, and substance use impeding activities of daily life are all symptoms noted in the DSM-5. Importantly, the DSM-5 includes language categorizing both tolerance (a common correlate of worsening withdrawal) and craving as meaningful symptoms of an Inhalant Use Disorder, but the present criteria do *not* include any other symptoms related to drug abstinence (e.g., anxiety, depression, seizures, etc.).

1.3: Toluene Epidemiology

Despite a serious risk of permanent harm or even death (Butland et al., 2012), the abuse-inhalation of compounds containing volatile organic solvents for their intoxicating properties remains a persistent health concern (Howard et al., 2011). Alarmingly, 22% of individuals found dead of an inhalant-related incident had no known history of inhalant abuse (J. F. Williams, Storck, Abuse, & Health, 2007), possibly suggesting that use can be hidden successfully and/or that even occasional inhalant abuse can be deadly, accidental or otherwise. Evidence suggests that the number of new inhalant abusers in the United States is approximately 750,000 individuals every year (Howard et al., 2011). In 2013, 6.3% of American teens who experimented with recreational drug use for the first time chose to initiate their drug use with inhalants (SAMHSA, 2014). The abuse of inhalants in the United States is concentrated in adolescent populations, with 10.8% of American 8th grade students admitting to having inhaled a solvent at least once in their lifetime with the intent of achieving intoxication (Johnston, O'Malley, Miech, Bachman, & Schulenberg, 2015). This is juxtaposed with an overall lifetime incidence of 6% (SAMHSA, 2014), suggesting

that solvent abuse experiences are clustered around early adolescence. Past year (5.3%) and past-month (2.2%) usage of inhalants among 8th graders is *lower* than lifetime use (Johnston et al., 2015). This is likely because inhalant abuse rates tend to decrease with age (oddly, 12th grade students show lower lifetime prevalence rates than 8th graders), however survey findings suggest an overall adolescent lifetime prevalence rate of 8.9% (Kann et al., 2014).

This prevalence is by no means homogeneous, however. For example, inhalant abuse has historically been more prevalent amongst males than females; however, more recent survey data indicates that this gap has closed (Kann et al., 2014). Indeed, the Youth Risk Behavior Surveillance System (YRBSS; Kann et al., 2014) national survey of students reported that lifetime prevalence of inhalant abuse is now higher in females (10%) than males (7.9%). This study also noted that inhalant abuse in the United States differs by ethnicity with Hispanic individuals (11.7%) showing greater lifetime use than white (8.6%) or black (6.8%) individuals. Additionally, some evidence suggests that Native American adolescents reported higher rates of inhalant abuse than white peers (Dieterich, Stanley, Swaim, & Beauvais, 2013).

Further, socioeconomic and geographic factors appear to play a role in inhalant abuse prevalence. For example, 17.9% of 12th graders in an impoverished rural region of Mississippi indicated some lifetime use (McDermott et al., 2013) as compared to 10% of adolescents in Mississippi as a whole and 6.9% of nationwide 12th graders from the same time period (Johnston et al., 2015; Kann et al., 2014). In fact, inhalant abuse also varies from state to state, with higher lifetime prevalence rates clustered in states in the rural south; e.g., 14.5% for Louisiana, 13.1% for Alabama, 13.1% for Arkansas, and 11% for Tennessee (Kann et al., 2014). Indeed, four of the five states with the highest lifetime adolescent inhalant abuse percentages are located in the rural southern United States (the exception being Wyoming, 11.1%, also a predominantly rural region).

It is impossible at this time to separate the influences of poverty, ethnic, community and other cultural factors, although multiple influences likely play a role in defining risk.

1.4: Pharmacological properties of toluene

Toluene is highly fat and lipid soluble with nearly 80% of inhaled toluene vapor reaching the blood stream (Donald, Hooper, & Hopenhayn-Rich, 1991). Unlike ETOH, toluene appears to follow first-order kinetics for metabolism (Bray, Thorpe, & White, 1950). Toluene is classified as a central nervous system (CNS) depressant, and like many such substances has concentration-dependent biphasic behavioral pharmacodynamics in humans and animals (Moser & Balster, 1985). Similar to low-level alcohol (ETOH) intoxication, humans report that subjectively "low-level" exposure to toluene vapor produces disinhibition and euphoria (Cruz, 2011; Siegel, Alvaro, Patel, & Crano, 2009). At higher concentrations, toluene produces classic CNS depressant effects including ataxia, confusion, slurred speech, unconsciousness, and death (Bowen, 2011). It is important to note that with inhalants like toluene, "dose" is more properly conceptualized as a composite measure of "magnitude of exposure" encompassing the concentration of toluene in the air, the frequency and depth of inhalations, and the duration of the bout. Evidence from research into the teratogenic properties of toluene indicates that a high external concentration of toluene alone is not necessarily sufficient to disrupt development (S. P. Callan, Kott, J.M., Cleary, J.P., McCarthy, M.K., Baltes, B.B., Bowen, S.E., 2015). Instead, a balance between the concentration of toluene ("dose"), the frequency and pattern of the exposures, and the duration of the exposures must be sought. That is to say, high-dose, short-duration exposure and low-dose, long-duration exposure may not be optimal methods of effecting physiology, depending of course on the physiological measure in question. Instead, the most effective exposure paradigms may be those that find a medium between high concentrations and long exposures. Toluene, like ETOH, appears

to have limited anxiolytic properties, at least while the user is intoxicated (Cruz, 2011). Unlike ETOH, there have been reports that acute toluene intoxication can result in dissociative hallucinations at higher doses (Cruz & Dominguez, 2011). As with ETOH, some reports exist suggesting that high doses of toluene and other solvents may cause convulsions (Flanagan & Ives, 1994).

Similar to ETOH (Criswell & Breese, 2005), toluene appears to have effects on several neurochemical sites, one of those being a potentiator of the inhibitory Gamma-Aminobutyric Acid ($GABA_A$) receptor. Indeed, electrophysiological studies in hippocampal tissue slices from rat brain revealed that acutely applied toluene potentiated $GABA_A$ receptor function (Beckstead, Weiner, Eger, Gong, & Mihic, 2000). Toluene has also been shown to depress hippocampal excitability, which was reversible with a $GABA_A$ receptor antagonist (MacIver, 2009), further suggesting GABAergic potentiation. This potentiation of GABA receptor function appears to be functionally relevant, as $GABA_A$ receptor antagonism blocks toluene-induced hypothermia (Paez-Martinez, Aldrete-Audiffred, et al., 2013). Shelton and Nicholson (2013) found that mice will substitute benzodiazepines for toluene vapor, further suggesting agonistic action at GABA receptors. Finally, toluene vapor has protective effects on the excitatory glutamatergic NMDA-induced seizures, typical of its role as a GABA receptor agonist (Cruz, Gauthereau, Camacho-Munoz, Lopez-Rubalcava, & Balster, 2003) alongside its action as glutamate antagonist on NMDAR (see below).

In addition to sharing GABA agonism, toluene and ETOH appear to share glutamate receptor antagonistic properties. Acutely applied toluene inhibits NMDA receptor function during exposure, but results in an increase in NMDA receptor functioning following the acute exposure (Bale, Tu, Carpenter-Hyland, Chandler, & Woodward, 2005). Like alcohol, repeated toluene exposure increases GluN1 and GluN2B sub-receptor protein expression in a variety of brain

regions, including the prefrontal cortex and nucleus accumbens (J. M. Williams, Stafford, & Steketee, 2005). Recent evidence demonstrates that toluene pre-exposure suppresses responding to NMDA antagonism, suggesting that toluene had disrupted NMDAR functioning (Duncan, Gibbs, & Lawrence, 2014). NMDA receptor antagonism appears to be behaviorally relevant, as pretreatment with d-serine (a glycine co-agonist) completely blocks toluene-induced changes to behavior including hyperactivity and memory impairment, suggesting that NMDA antagonism plays a critical role in toluene's behavioral effects (e.g., Lo, Wu, Sue, & Chen, 2009). As has been mentioned previously, however, the rewarding effects of toluene exposure are *not* mediated by NMDA receptor antagonism, though evidence suggests that memory and locomotor effects of toluene exposure are recovered by NMDA co-agonism (Chan, Chung, Stoker, Markou, & Chen, 2012). NMDAR antagonism is an important mechanism for characterizing toluene withdrawal because it has been suggested that increases in seizure activity seen during ETOH withdrawal are mediated by the increase in glutamate activity at NMDA receptors (Crabbe, Kendler, & Hitzemann, 2013), most likely mediated by increased limbic system irritability (Jasova, Bob, & Fedor-Freybergh, 2007) brought on by the kindling effect, an adaptive response to repeated bouts of intoxication and abstinence such that a cycle of drug use and withdrawal results in more severe subsequent withdrawal (for a review, seeBallenger & Post, 1978).

The rewarding effects of toluene inhalation (Bowen, Batis, Paez-Martinez, & Cruz, 2006) appear to be mediated by the mesolimbic dopamine (DA) system (Riegel, Zapata, Shippenberg, & French, 2007). Acute exposure to toluene vapor increases c-Fos mRNA expression in the rat nucleus accumbens (NAc;Perit et al., 2012), while repeated toluene exposure increases NAc c-Fos in mice (Tomaszycki, Aulerich, & Bowen, 2013). This suggests that toluene vapor increases activity at this brain region which is typical of rewarding drugs. Lesions of the NAc result in the

abolishment of toluene-induced hyperactivity (Riegel, Ali, & French, 2003). Electrophysiological evidence indicates that toluene exposure increases DA neuron activity in the ventral tegmental area (VTA) suggesting that toluene exposure activates the mesolimbic reward pathway (Riegel & French, 2002). Furthermore, application of toluene to the rat VTA results in an increase in DA release in the NAc, clear evidence of drug-mediated reward (Riegel et al., 2007). Increased extracellular DA in the rodent NAc during toluene exposure has been replicated by other research groups, and has been linked to increased locomotor activity during exposure (Stengard, Hoglund, & Ungerstedt, 1994).

CHAPTER 2: INHALANT WITHDRAWAL

Despite potent neurochemical effects, clinical reports of inhalant withdrawal have been sparse. Some clinical researchers counsel healthcare providers that inhalant withdrawal is rare enough (and mild enough) to not warrant pharmacological intervention (Patel, 2014). This decision does not appear to be based on any scientific consensus. The DSM-5 criteria for inhalant use/dependence do not include withdrawal symptoms in the Inhalant Use Disorder diagnosis of inhalant dependence (American Psychiatric Association, 2013) despite calls during the formulation for the DSM-5 to include withdrawal alongside tolerance and other symptoms of growing physiological dependence (Perron, 2009; 2011). It is worth noting, however, that the DSM-5 does include increased craving for inhalants as a diagnostic symptom, which many researchers and clinicians include as a feature of (psychological) withdrawal (Ridenour, Bray, & Cottler, 2007; Shah et al., 1999).

Critically, the opinion that inhalant withdrawal is an insufficient criterion of inhalant dependence is *not* borne out in the clinical data. A survey of the population served by a tertiary care clinic in South India indicated that 100% of individuals classified as "Inhalant only" abusers displayed withdrawal symptoms, though specific symptoms were not catalogued (Narayanaswamy, Viswanath, Ravi, & Muralidharan, 2012). Additionally, these authors found that individuals who admitted to polydrug use including inhalants displayed significantly *lower* incidences of inhalant withdrawal, possibly indicative of cross-dependence or some other interaction between inhalants and other classes of abused drug. This may help to explain the mixed reporting of inhalant withdrawal pathology. A similar study conducted at a substance abuse clinic, also in India, reported physiological inhalant withdrawal symptoms in over half of participants surveyed and nearly all reported strong craving arising during abstinence (Kumar et al., 2008).

Ridenour and colleagues (2007) examined the test-retest reliability of the DSM-IV criteria for inhalant dependence in an American adolescent cohort and found that those criteria showed poor reliability. Specifically, they noted that more than one in ten of the adolescents surveyed reported "withdrawal symptoms" (the second most commonly endorsed experience of these inhalant users), which suggests that inhalant withdrawal is indeed a meaningful symptom of dependence. It is worth noting that the DSM-IV did not include craving as a significant symptom of inhalant dependence (or any other substance use disorder), though this criterion was added to the DSM-V. This evidence is further supported by numerous case-reports suggesting that individuals who habitually abuse inhalants report experiencing classic symptoms of withdrawal such as anxiety and drug craving typically within a day of abstinence (Keriotis & Upadhyaya, 2000; Kouzoupis et al., 2010; Muralidharan, Rajkumar, Mulla, Nayak, & Benegal, 2008; Niederhofer, 2007; Shen, 2007). Many of these case-reports demonstrate that pharmacological interventions commonly utilized to mitigate alcohol withdrawal symptoms are an efficacious method of treating withdrawal and postponing relapse to drug behavior. In a study of newborns with recent prenatal exposure to inhalants (the mother smelled of solvents upon arrival for childbirth), 75% met diagnostic criteria for substance withdrawal brought on by passive substance addiction (e.g., high pitched cry, sleeplessness, hyperactive Moro reflex and tremor; Tenenbein, Casiro, Seshia, & Debooy, 1996). Contrasting the alcohol literature, these authors report that the onset of the withdrawal symptoms was *later* than what is typically observed in neonates who display alcohol withdrawal, likely due to differences in the pharmacokinetics of these substances.

As for the symptoms of inhalant withdrawal syndrome, there does seem to be consensus in the literature. In their examination of inhalant dependence, Ridenour and colleagues (2007) report that headaches, vomiting, craving, depressed mood, and anxiety are seen reliably during

abstinence. In a report detailing adolescent gasoline abusers, Shah et al., (1999) reported anhedonia, irritability, craving, headache, and psychomotor retardation as primary symptoms during abstinence. These authors note that the withdrawal symptoms they observed were similar to symptoms observed during alcohol withdrawal. Kumar and colleagues (2008) reported withdrawal symptoms to inhalants including irritability, restlessness, and headache. Perron and colleagues (2011) reported a wide variety of symptoms including hypersomnia, depressed mood, nausea, tachycardia, anxiety, headaches, hallucinations, tremors and even seizures to withdrawal from inhalants.

The signs and symptoms observed during inhalant withdrawal appear to be consistent with what has been documented for withdrawal from alcohol (ETOH) and other central nervous system depressants. In fact, a study of Japanese individuals with a substance use disorder diagnosis found that alcohol and inhalants produced qualitatively similar physical withdrawal symptoms (tachycardia, perspiration, shivering, appetite change, and tremor), though the authors note that in most cases alcohol withdrawal produces significantly stronger symptoms (Kono et al., 2001). Additionally, these authors report significant cognitive-affective signs of withdrawal amongst inhalant abusers (e.g., drug craving, anxiety & restlessness), similar to, though again less severe than, what has been reported during alcohol withdrawal.

There is a consensus in the alcohol (ETOH) literature that withdrawal symptoms are largely mediated by alterations to NMDA and GABA function that occur subsequent to chronic alcohol abuse (Cooper & Vernon, 2013 for a review). The increase in glutamatergic excitation that arises following chronic abuse persists after alcohol intoxication ends, giving rise to adverse symptoms (Lovinger, 1993 for a review). Given that inhalants (such as toluene) share critical pharmacodynamic properties with alcohol, it seems plausible that a mechanism of action for many

inhalant withdrawal symptoms following chronic toluene abuse may also be glutamatergic excitation secondary to NMDAR blockade (Garland & Howard, 2012; Lubman, Yucel, & Lawrence, 2008), the same effect that drives many alcohol withdrawal symptoms (Chen, Jarrott, & Lawrence, 1999). Indeed, these mechanisms could easily be assessed via tissue content analysis or protein assay once a working model of inhalant withdrawal was established.

As was previously mentioned in Chapter 1, a drawback of survey data regarding inhalant use is the tendency to group chemicals with differing pharmacodynamic properties together into one class ("Inhalants"). Few studies have attempted to differentiate withdrawal symptoms between types of inhalants, largely due to concerns with sample size and limitations on questions asked. There is limited evidence for toluene-specific features in an inhalant abstinence syndrome. First, a report of toluene abusers suggests that 70% developed increased tolerance to toluene (A. C. Evans & Raistrick, 1987). The incidence of tremor, at least, is corroborated by a case-report of a man exposed accidentally to low-dose, high duration toluene vapor in an industrial setting who displayed tremor like symptoms following his removal from the exposure source (Mills, Grigg, Offermann, Gustin, & Spingarn, 2012).

An additional limitation to survey data concerning inhalant abuse is a lack of stratification of participants by severity of inhalant abuse. When subjects are stratified by DSM-IV diagnostic criteria (e.g., use, abuse, dependence), a pattern becomes clear: 12.2% of inhalant users report withdrawal symptoms compared to 43% of abusers and 77.3% of dependent individuals (Perron et al., 2011). This suggests a clear escalation of withdrawal severity, with longer-term, habitual inhalant abuse resulting in more reliable incidences of withdrawal. In Perron and colleagues' study (2011), individual symptoms follow a clear upward progression as well. Individuals with a DSM-IV diagnosis of inhalant abuse disorder displayed significantly lower levels of abstinence-induced

anxiety (17.4%) than those classified as having an inhalant dependence disorder (41.8%). The same is true for depression (17.2% and 42.2%, respectively) and tremors (9.8% and 37.8%). While this study demonstrates that inhalant withdrawal symptoms occur in similar percentages to cocaine (their reference drug), the authors note that only 10.3% of inhalant-dependent individuals reported using inhalants to avoid withdrawal symptoms, while nearly half of cocaine-dependent individuals reported withdrawal-avoidance use. The authors stress that this is a qualitative assessment, as direct statistical comparison was impossible due in part to skewed sample size (cocaine-dependent subjects outnumbered inhalant-dependent subjects 18:1). It is possible that the 10.3% of inhalant-dependent individuals are substantially more advanced in their inhalant dependence than their peers. It is also possible that cross-dependence from poly-drug use may be suppressing withdrawal symptoms in this sample (e.g., Narayanaswamy, et al., 2012).

There is a dearth of animal studies regarding the mechanistic understanding of inhalant withdrawal, despite the utility of preclinical models in confirming symptoms reported in human surveys and providing potential mechanisms for these symptoms. There has been no published investigation of symptoms of toluene withdrawal, either behaviorally or neurochemically, in animals. Only one study explicitly investigated withdrawal symptoms from the solvent 1,1,1-trichloroethane (TCE) which were reported to include lowered seizure threshold and potentiated handling-induced seizures in rodents (E. B. Evans & Balster, 1993).

2.1: Towards an animal model of toluene withdrawal

To assess the existence of toluene withdrawal pathology in rodents, it is necessary to develop a method of reliably and humanely inducing toluene withdrawal. Indeed, the conclusion of the existence of a physiological “withdrawal” syndrome for toluene abuse is predicated upon first demonstrating that animals display altered biobehavioral responses during a period of toluene

abstinence. For this, it is reasonable to follow the example of previously published solvent withdrawal / abstinence studies. Evans and Balster (1993) examined the effects of 1,1,1-trichloroethane (TCE) on withdrawal-mediated seizure activity. They exposed mice to one of several behaviorally relevant doses of TCE for 96 consecutive hours. This is similar to alcohol withdrawal studies in which mice were exposed to ETOH vapor for 3 or more days (e.g., Crabbe et al., 2013). However, a complication is immediately apparent when comparing toluene to TCE and ETOH: toluene is much more potent than either of these solvents (Moser & Balster, 1985). As a result, a 72- or 96-hour toluene exposure may have unintended deleterious consequences beyond the occurrence of withdrawal (weight loss, soft tissue irritation, respiratory suppression, etc.). Thus, it is necessary to first find the *least* amount of time required to induce toluene withdrawal. As such, it is logical to initially pursue an acute withdrawal paradigm, whereby a single, relatively short (e.g., 24 hours) massed exposure to the drug is given, rather than many repeated exposures. Such models exist in the ETOH literature (Kosobud & Crabbe, 1986) that can serve as a guide.

A second important factor for designing a toluene-specific model of withdrawal is identifying the ideal time to test behaviors. Toluene is rapidly eliminated from blood (Bruckner & Peterson, 1981) in a curvilinear manner consistent with first-order kinetics, and the half-life of toluene in the mouse is estimated to be as low as 25 minutes (Koga, 1978), suggesting that the majority of toluene is eliminated from the body within two to three hours. A more clinically relevant functional parameter, however, may be measuring when toluene-exposed animals regain normal ambulatory function. Bruckner and Peterson (1981), in their seminal two-part examination of toluene's pharmacokinetic and pharmacodynamic properties, noted that the abolishment of toluene's narcotic-like properties coincides with the elimination of toluene from brain tissue, which occurs slightly faster than toluene is eliminated from blood. Despite this, there has been no

study examining the return of normal behavioral functions in animals exposed to prolonged abuse levels of toluene vapor. Therefore, should toluene-exposed animals display symptoms of sedation upon removal from the exposure chamber, an important step in the process of developing a model of toluene withdrawal is to ascertain at what point toluene-exposed animals regain control of motor function as assessed by regaining the righting reflex. This can be done in parallel with the examination of seizure activity, which can be measured even when animals are still sedated. A logical step would be to track the emergence of handling-induced seizures following a prolonged exposure to toluene vapor as this assessment can be performed repeatedly without confounding the outcome. The 96-hour prolonged exposure model (e.g., E. B. Evans & Balster, 1993) is a natural starting point. A locomotor-stimulating exposure of toluene (e.g., 1000 ppm - 5000 ppm) should be utilized to reduce the risk of acute toxicity. If this exposure scheme proves insufficient to induce seizures, the next step would be to modify a chronic intermittent exposure model to induce the kindling effect. If this experiment proves deleterious to the health of the animals, however, as previously discussed a balance between concentration and duration must be sought.

Once a working method of inducing toluene-abstinence is achieved, the next step is to establish the validity of this method. While the presence of handling-induced seizures provides qualitative evidence for a withdrawal state, quantitative measures may prove helpful. As such, a second method of assessing changes in seizure threshold, such as by the administration of a pro-convulsant, will provide concurrent validity to the previous finding. Unlike the handling-induced model, where the expected variance of the control group is zero (and thus statistics cannot be performed), PTZ-induced seizures should allow for statistical hypothesis testing. Next, demonstrating that this method will produce bio-behavioral changes beyond alterations to seizure threshold will provide convergent validity. As has been previously noted, anxiety is a common

symptom of abstinence reported by inhalant abusers. As such, establishing that this method produces an anxious phenotype in mice would increase our confidence in this measure. Finally, a measure of face validity is essential. A core principle of drug withdrawal is that taking more drug is a method of relieving withdrawal symptoms. As such, toluene-abstinent animals should see an amelioration of their symptoms if re-exposed to toluene vapor.

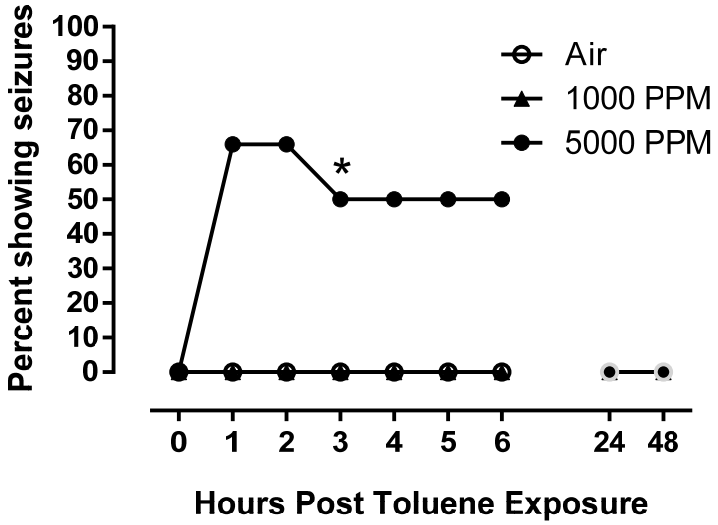

Figure 1: PILOT DATA. The effect of protracted toluene exposure on handling-induced seizures. This data represents pilot testing of prolonged (96-hour) toluene exposure followed by 48 hours to behavioral testing. Note that for 1000 ppm, N = 10, while initial N = 4 for 5,000 ppm. Animals in the 1000 ppm condition were exposed continuously for 96 hours, while exposure in the 5,000 ppm condition was stopped at 72 hours when one of the animals was found dead. *At three hours post-exposure, one of the three remaining 5,000 ppm animals was euthanized. All subsequent measures were made with N = 2 for 5,000 ppm.

Initial pilot testing was intended to expose animals to 5,000 ppm toluene vapor for 96 consecutive hours. However, one of the four toluene animals died during the second night of exposure. We decided to discontinue toluene exposure after 72 hours to preserve the safety of the remaining animals. Even so, the remaining three animals showed signs of soft-tissue irritation (swollen, irritated eyes and nose). A second animal in the toluene group was euthanized during seizure testing as a direct consequence of these symptoms. We repeated this protocol, reducing the

concentration of toluene to 1,000 ppm. All 10 toluene animals completed the 96-hour exposure with no adverse effects, however, none of the animals displayed handling-induced seizures. Based on these results, we resolved to reduce the duration of the exposure to 5,000 ppm for 24 hours, a time period when none of the toluene-exposed animals displayed signs of soft tissue irritation.

After completing pilot testing, the first logical step in characterizing withdrawal from toluene was examining the effect of toluene withdrawal on handling-induced seizures. This allowed me to establish a timeline for alterations in seizure susceptibility that arose during abstinence. This timeline was used to select an optimum window of time for more direct pharmacological manipulation. Once such a window was established, I next sought to determine whether toluene withdrawal altered the severity of seizure responses elicited by an injection of a known pro-convulsant (pentylenetetrazol). This provided both statistically quantifiable data and allowed me to assess a second profile of toluene's action on seizure susceptibility during withdrawal. Following this, I sought to determine whether toluene withdrawal would lower seizure thresholds by producing seizures to a sub-threshold injection of pentylenetetrazol and what effect re-exposure to toluene vapor would have on this responding. Both of these concepts are classic hallmarks of CNS depressant withdrawal.

For assessing the impact of toluene withdrawal on anxiety, I first started with the gold standard Elevated Plus Maze. Following this, I assessed whether re-exposure to toluene would ameliorate the effects of toluene withdrawal. To build upon the construct validity of my model, I repeated this test utilizing the Light/Dark box, Open Field Test, and Marble Burying Test. The Open Field Test had the added benefit of allowing a direct examination of the locomotor effects of toluene withdrawal, while the Marble Burying test provided a measure of anxiety unrelated to preference behavior. As these three subsequent tests have never been utilized in the study of

toluene withdrawal before, I also examined the putative anxiolytic effects of an acute exposure to toluene in these assays. Finally, the Elevated Plus Maze, Light Dark Box, and Marble Burying Test were new protocols for our group, and as a result I first validated these measures using an acute ethanol condition. As such, the specific research questions and hypotheses are listed below.

2.2: Research Questions and Hypotheses

1. *Research Question:* Will a 24-hour acute exposure be sufficient to produce handling-induced seizure activity in mice?

    a. **Hypothesis 1:** Abstinence following 24-hour acute massed exposure to toluene vapor will produce handling-induced seizures in mice.

2. *Research Question:* Will mice exposed to 24-hour acute massed exposure to toluene vapor demonstrate reduced seizure threshold, typified by a more severe seizure response compared to air controls, in response to an injection of a pro-convulsant agent?

    a. **Hypothesis 2:** Abstinence following acute massed exposure to toluene vapor will increase the severity of seizures induced by 48 mg/kg pentylenetetrazol (PTZ) injection given 3 hours after termination of toluene exposure.

    b. **Hypothesis 3:** Mice abstinent from acute massed exposure to toluene vapor following a 24-hour exposure will display seizure activity to a 42 mg/kg sub-threshold PTZ injection, while control animals will not.

3. *Research Question*: Will abstinence following acute massed toluene exposure increase anxiety-associated behavior(s) in mice?

    a. **Hypothesis 4:** Mice abstinent from acute massed exposure to toluene following a 24 hour exposure will demonstrate an anxious phenotype as

typified by decreased proportional open arm exploration on the elevated-plus maze at 24 hours of abstinence, but not 72 hours of abstinence.

b. **Hypothesis 5:** Mice abstinent for 24, but not 72, hours from acute massed exposure to toluene following a 24-hour exposure will demonstrate an anxious phenotype as typified by increased thigmotaxis (movement along the outer rim of an enclosure) in an Open Field Test.

c. **Hypothesis 6:** Mice abstinent for 24, but not 72, hours from acute massed exposure to toluene following a 24-hour exposure will demonstrate an anxious phenotype as typified by increased proportional preference for the dark chamber in a Light/Dark Box test.

d. **Hypothesis 7**: Mice abstinent for 24, but not 72 hours from acute massed exposure to toluene following a 24-hour exposure will demonstrate an anxious phenotype as typified by increased marble burying in the Marble Burying Task.

4. *Research Question:* Will re-exposure to toluene vapor ameliorate the behavioral symptoms that arise during toluene abstinence?

a. **Hypothesis 8:** 30-minute re-exposure to acute massed exposure to toluene vapor will reduce the increased sensitivity to PTZ injections in abstinent mice as well as blocking the anxiogenic properties of toluene abstinence.

CHAPTER 3: METHODS

3.1: Subjects

Adult male Swiss Webster mice (N = 412) obtained on Post-Natal Day (PND) 30 were used in this experiment. This strain was chosen to conserve similarities between this work and the only prior examination of inhalant withdrawal (Evans and Balster, 1993) especially with the knowledge that various mouse strains have differential effects to toluene (Bowen, Kimar, & Irtenkauf, 2010). We chose to exclusively use adult mice because toluene is known to have differential effects on adolescent animals (Batis, Hannigan, & Bowen, 2010; Bowen, Charlesworth, Tokarz, Wright, & Wiley, 2007), and exclusively male mice to control for confounds due to hormone changes. Mice were purchased from Envigo RMS (Indianapolis, IN, USA). The Institutional Animal Care and Use Committee at Wayne State University approved all animal procedures and behavioral experiments. Procedures were conducted in accordance with the NIH "Guide for the Care and Use of Laboratory Animals: Eighth edition" (National Academy of Sciences, 2011, revised 2010). Animal numbers are listed in Table 1.

Table 1: Animal numbers

Hypothesis	**Test**	**Toluene**	**Duration**	**Tested**	**Projected N**
Hypothesis 1	Handling Induced Seizures	Air	24 hours	Hourly	N = 8
		5,000 ppm	24 hours	Hourly	N = 8
					Total = 16
		Air	24 hours	3h post	N = 18

Hypothesis 2	48mg/kg PTZ Induced Seizures	5,000 ppm	24 hours	3h post	N = 18
					Total = 36
Hypothesis 3	42mg/kg PTZ Induced Seizures	Air	24 hours	3h post	N = 10
		5,000 ppm	24 hours	3h post	N = 10
					Total = 20
Hypothesis 4	Elevated Plus Maze	Air	24 hours	24h post	N = 10
				72h post	N = 10
		5,000 ppm	24 hours	24h post	N = 10
				72h post	N =10
		5,000 ppm	30 minutes	Immediately	N = 10
		Air			N = 10
		Ethanol	Injection	5 minutes later	N = 10
		Saline			N = 10
					Total = 80
Hypothesis 5	Open Field Test	Air	24 hours	24h post	N = 10
				72h post	N = 10
		5,000 ppm	24 hours	24h post	N = 10
				72h post	N = 10
		5,000 ppm	30 minutes	Immediately	N = 10
		Air			N = 10

					Total = 60
Hypothesis 6	Light/Dark Box	Air	24 hours	24h post	N = 10
				72h post	N = 10
		5,000 ppm	24 hours	24h post	N = 10
				72h post	N = 10
		5,000 ppm	30 minutes	Immediately	N = 10
		Air			N = 10
		Ethanol	Injection	5 minutes	N = 10
		Saline			N = 10
					Total = 80
Hypothesis 7	Marble Burying	Air	24 hours	24 hours after	N = 10
		5,000 ppm			N = 10
		Air			N = 10
		5,000 ppm	24 hours	72 hours after	N = 10
		Air	30 min	Immediately	N = 10
		5,000 ppm			
		Saline	Injection	5 minutes	N = 10
		Ethanol			N = 10
					Total = 80
Hypothesis 8	Toluene Re-exposure	Air	24 hours	30m before 42mg/kg PTZ	N = 10
		5,000 ppm			N = 10
		Air		30m before 24h EPM	N = 10
		5,000 ppm			N = 10

					Total = 40
TOTAL					N = 412

3.2: Exposure Apparatus

Dynamic solvent exposure systems (e.g., Bowen, Wiley, & Balster, 1996) make use of constant airflow in an unsealed chamber. A constant concentration of vapor within the chamber was achieved by matching the input flow-rate with the natural outflow of the chamber itself. The dynamic exposure system utilized for these experiments consisted of a 37.8 L glass rectangular tank (51cm x 28cm x 12cm, 1428 cm^2 floor area) fitted with a Plexiglas® lid into which toluene was delivered in the airflow. The lid was equipped with 1.2 cm circular access ports located at opposite ends which allowed for delivery of toluene vapor.

Toluene vapor was generated by filtering air into a bubbler immersed in a 500-ml solvent bath contained in a 1-L round-bottom flask. Air saturated with vapor exited the bath and mixed with fresh air from a second line before being delivered to the exposure chamber. By adjusting the ratio of vapor-laden air and filtered air, the rate through which toluene entered the chamber could be adjusted and the concentrations held constant (See Figure 2). Concentrations in the chamber were monitored using a single wavelength monitoring infrared (IR) spectrometer (Miran 1A, Foxboro Analytical, North Haven, CT). The IR spectrometer input tubing was inserted into the testing chamber through a second access port in the lid.

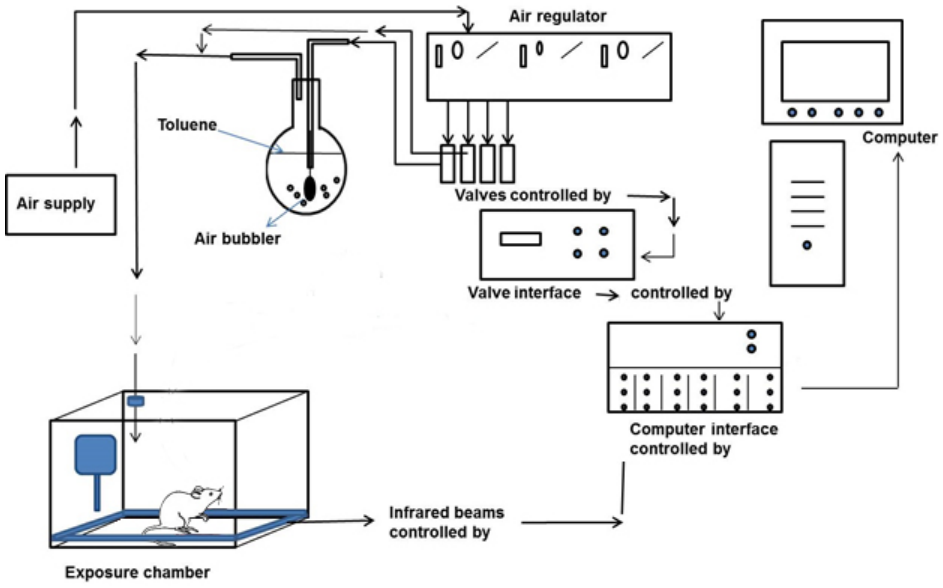

Figure 2: Schematic representation of the dynamic toluene exposure system utilized in this protocol.

For these experiments, the chamber floor was covered with fresh bedding. A small portion of the animals' home-cage nests were placed in the corner furthest from the toluene inlet port to reduce the stress of the novel environment. Fresh food was placed adjacent to the nest and a water bottle was affixed to the opposite wall. Animals in the air control condition had identical exposure chambers, save that the air entering their chambers lacked toluene vapor. Animals were exposed in groups of 10, except for the 30-minute acute exposures in Hypotheses 4-6 which were singly housed during the half-hour toluene exposure. Local lighting was set on a 12-hour light/dark timer with lights on at 0700. Animals were kept on a traditional light/dark cycle to facilitate behavioral observations. The testing area was temperature controlled to 20-22°C with humidity between 40-70%.

3.3: Acute Toluene Abstinence

On the day of exposure, two groups of mice (N = 10/group) were brought to the laboratory at 0900 h for a 30-minute habituation period. Once habituated, the mice were weighed, tail-marked

using a felt-tipped permanent marker, and placed into the exposure chamber or an identical air control chamber to habituate for 30 minutes to the chambers. Following habituation, the exposure period commenced and animals were exposed to either 0 ppm or 5,000 ppm toluene vapor. The 5,000 ppm concentration and the 24-hour exposure duration for toluene was chosen following pilot testing (see Figure 1), as it represented a concentration that was not locomotor suppressing (Bowen & Balster, 1996, 1998). Pilot testing suggested that a balance between the high concentration (5,000 ppm) and longer exposure time (e.g., 96 hours) would be efficacious without causing harm, so a high-dose of 5,000 ppm and moderate exposure time model 24hr was selected. Animals were observed directly hourly starting at 1000 h and continuing until 1800 h. The next morning, toluene exposure was discontinued at 1000 h (i.e., 24 h later). Animals were then removed from the exposure chambers and returned to their home cages. Animals who lacked a righting reflex upon removal from the testing chamber were placed on a paper towel to reduce the risk of aspiration of bedding. With the exception of Hypothesis 1, all animals were monitored for at least 3 hours before commencing behavioral studies. Animals used for testing hypotheses 4-8 were monitored for 3 hours before being returned to home cages until testing (at either 24 or 72 hours post-exposure). We had originally proposed to eliminate any animals whose weight dropped below 80% of pre-exposure values as well as any animals that displayed signs of soft-tissue irritation (e.g., swelling, rash, or redness around the eyes or nose), but no such cases occurred during the study.

3.4: Specific Methods

3.4.1: H1: Handling-induced seizures in mice following toluene exposure

To assess the time-course of seizure activity during toluene abstinence, a handling technique was utilized to assess changes in seizure threshold (e.g., the "tail twist test"). Immediately after toluene exposure and continuing every hour for 6 hours (and again at 24 and 48

hours), mice (N = 16) were manually picked up by the tail and gently twisted or spun to assess for handling induced seizures. The excitation brought on by handling often triggers seizure activity in mice with lowered seizure thresholds (but not in normal control animals). This is modeled after the protocol published by Balster and colleagues (1993) demonstrating that animals at increased risk of seizure produced tremor movements following this motion. This model has been validated in studies of alcohol withdrawal as well (Becker, Diaz-Granados, & Weathersby, 1997; Becker & Hale, 1993). Mice were video recorded during testing. Seizure activity was scored by a trained observer using the guidelines detailed in the Functional Observational Battery developed by Balster and colleagues (Bowen, Wiley, Evans, Tokarz, & Balster, 1996; Tegeris & Balster, 1994). A second rater, blind to treatment condition, verified all scores. Discrepancy between the raters was handled by consultation between the raters. The Functional Observational Battery breaks seizure activity into clonic and tonic movements, and lists valid behaviors within each category. Raters observe the animals and indicate the presence or absence of behaviors in either category. Overall severity is determined by the presence of clonic and tonic behaviors, with the presence of tonic behaviors being considered a more severe reaction. **Clonic movements** are typified by the alternate contraction and relaxation of muscles, usually presenting as a tremor or shaking motion. **Tonic movements** are typified by the prolonged and involuntary contraction or rigidity of muscles. Animal seizure behavior falls along a progression of severity that can be broken into five groups: no seizure, clonic symptoms only, a combination of tonic and clonic behaviors (tonic-clonic), tonic behaviors alone, or a lethal seizure. The behaviors that constitute clonic or tonic movements are listed on Table 2. This method has been validated by numerous studies. Alcohol withdrawal increases the severity and potential lethality of convulsants (Becker, 1998; Becker, Veatch, & Diaz-Granados, 1998). In addition to alcohol, TCE withdrawal has been shown to produce

handling-induced seizures as well as to increase the severity of seizures induced by convulsant injection (Evans and Balster, 1993). Note that for Hypothesis one, due to the mild nature of the stimulus, seizure data was condensed to a dichotomous Seizure / No Seizure outcome.

3.4.2: H2 and H3: Pentylenetetrazol (PTZ)-induced convulsions in mice following 3-hour toluene abstinence

In this model, the animal was given a single injection of the convulsant at a dose that induced mild clonic tremors in control animals. A new cohort of mice (N = 10/ group) was exposed to either air or 5,000 ppm toluene for 24 hours. Following a 3-hour abstinence period, the mice were given a single injection of PTZ (42 mg/kg or 48 mg/kg) before being placed in solitary cage with a bedding floor. These doses were chosen based on prior literature in which 42 mg/kg has been shown to be a subthreshold dose in mice, while 48 mg/kg induces mild clonic tremors in control animals (Evans and Balster, 1993). The animals were recorded for a total period of 30 minutes because prior evidence suggested that peak seizure time occurs $\leq$15 minutes (Evans and Balster, 1993). Initially, the animals were observed to ensure that resulting seizures were not life-threatening. Later, a pair of researchers (blind to the treatment condition) watched the videos and the documented seizures were categorized by severity according to the same guidelines in Hypothesis 1. As in Hypothesis 1, any discrepancies between the two raters was resolved by consultation between the raters. Symptoms that constituted different seizure-categories (clonic, tonic-clonic, tonic, and lethal) are outlined in the Functional Observational Battery (Bowen et al., 1996). Animals displaying only clonic symptoms were scored as “clonic.” Animals displaying only tonic symptoms were scored as “tonic.” Animals displaying both tonic and clonic symptoms were scored as “tonic clonic.” Animals with seizures that resulted in death were scored as “lethal.”

Table 2: List of symptoms scored as seizure activity

Clonic Movements	Tonic Movements
(1)*Normal Appearance	(1)* Normal appearance
(2)*Quivers of limbs, ears, head, or skin	(2) Rigid, extended limbs (including tail)
(3) Repetitive movements of mouth and jaws	(3) Head and body rigidly arched back
(4) Mild tremors	(4) Head and body rigidly extended forward
(5) Severe or whole body tremors	(5) Explosive jumps into the air
(6) Wet dog shakes	(6) Severe convulsions
(7) Myoclonic jerks	
(8) Clonic convulsions	

Table 2: Behaviors scored as either clonic or tonic seizure movements. *These symptoms are scored as "no seizure activity." This table has been adapted from Tegeris and Balster (1994).

3.4.3: H4: Elevated Plus Maze test for anxious phenotypes

This experiment utilized six groups of animals: two groups of animals exposed to 24 hours of 5,000 ppm toluene, two groups exposed to 24 hours of air, one group exposed to 30 minutes of air, and one group exposed to 30 minutes of 5,000 ppm toluene (all N = 10/group; total = 60). The 24-hour exposure animals were tested on the Elevated Plus Maze at either 24 or 72 hours post-exposure, while the 30-minute exposure animals were tested immediately following exposure (positive control). Briefly, following the exposure/abstinence periods mentioned above mice were placed on a raised apparatus that consists of four arms (two walled [35 cm long X 5 cm wide X 15 cm deep] and two open [35 cm X 5 cm]). The animal was placed in the center of the maze and allowed to freely explore for 10 min. The animal was video recorded with motion-tracking software (Ethovision XT, Noldus). The relative amount of time spent on the open arms versus the closed arms was considered a measure of anxiety behavior, with an anxious phenotype presenting

as less time spent in the open arms (Pellow, Chopin, File, & Briley, 1985). Additionally, the total distance traveled and number of transitions into the open arms were used as measures of activity. This model has been validated in both rat (Pellow et al., 1985) and mouse (Lister, 1987) models as a reliable indicator of an anxious phenotype in rodents. Rodents in acute alcohol withdrawal display increased anxiety-like behavior as measured by increased time spent in the closed arms of the maze (Valdez et al., 2002). The acute 30-minute 5,000 ppm toluene exposure condition served as a positive control, as previous work has shown that acutely applied toluene is anxiolytic in mice at higher concentrations above and below 5000 ppm (Bowen, Wiley, & Balster, 1996). These animals were placed on the plus maze immediately following 30-minute toluene (or air) exposure. As this was a novel method for our laboratory, a second positive control was employed. A group of 10 mice were injected with 2 g/kg ETOH (i.p.) five minutes before introduction to the maze. A control group received i.p. saline.

3.4.4: H5: Open Field test for anxiety-like behavior

This experiment utilized six groups of animals: two groups of animals exposed to 24 hours of 5,000 ppm toluene, two groups exposed to 24 hours of air, one group exposed to 30 minutes of air, and one group exposed to 30 minutes of 5,000 ppm toluene (all N = 10/group; total = 60). Mice in the 24-hour exposure groups underwent testing at either 24 or 72 hours post-exposure. Mice (N = 10/group) were individually placed into an open chamber (28 cm X 28 cm x 29 cm high) with plastic walls and smooth steel flooring. The chamber itself was placed inside a sound-attenuating box during testing. The animal was allowed to freely explore the chamber for 30 minutes. Locomotor activity was measured via sets of photocells that bisected the transparent open field chamber. Interruptions of these photo beams results in analog signals being delivered by the photocells, which in turn triggers counters collected in one-minute bins during each procedure. The

computer was programmed to recognize an "inner" area (14 cm x 14 cm). The 30-minute exposure period was summed and the proportion of time spent in the center vs periphery was calculated. Anxiety-like behavior was typified by a lower proportion of time spent in the center area. As this is a locomotor-dependent measure, total distance traveled was also recorded as a validity control. As with Hypothesis 4, a pair of groups served as a positive control, receiving 30 minutes of 5,000 ppm toluene vapor or air immediately prior to introduction into the maze.

3.4.5: H6: Light/Dark Box test of anxiety-like behavior

This experiment utilized six groups of animals: two groups of animals exposed to 24 hours of 5,000 ppm toluene, two groups exposed to 24 hours of air, one group exposed to 30 minutes of air, and one group exposed to 30 minutes of 5,000 ppm toluene (all N = 10/group). Mice in the 24-hour exposure groups underwent testing at either 24 or 72 hours post-exposure. For this experiment, animals were placed in a polypropylene box (44×21×21 cm) unequally divided into two chambers (dark & light) separated by a wall with a small opening (8.5×5 cm). Each mouse was placed in the brightly lit (via a 400 lux LED puck light mounted to the roof of the test box) side of the box furthest from the dark chamber and given 10 min to explore. Automatic measurements of locomotion, rearing and time spent in light and dark zones and shuttle crossings between zones were recorded. Proportionally greater time spent in the dark zone and or a decreased latency to first entering the darkened zone indicates greater anxiety. The number of zone transitions and the distance traveled in the lit side of the chamber served as ambulatory controls. As in Hypothesis 4, because this method was new to our lab a second positive control was employed to validate the LDB. Mice (N = 10 / group) were given a single i.p. injection of either 2 g/kg ETOH or saline five minutes before introduction into the LDB.

3.4.6: H8: Marble Burying Test of Anxious- / Obsessive-like behavior

This experiment utilized six groups of animals: two groups of animals exposed to 24 hours of 5,000 ppm toluene, two groups exposed to 24 hours of air, one group exposed to 30 minutes of air, and one group exposed to 30 minutes of 5,000 ppm toluene (all N = 10/group; total = 60). The 24-hour exposure animals were tested on the Marble Burying Task at either 24 or 72 hours post-exposure, while the 30 minute exposure group was tested immediately following exposure (positive control). Briefly, at the appropriate time (see above) mice were individually placed into a plastic chamber (27 x 16 x 23 cm) containing 8 cm of lightly tamped bedding on which rested twenty 15-mm glass marbles evenly placed in a 5 x 4 grid. Mice were left in testing chambers for 30 minutes which were located in a separate enclosed room. Following testing, two researchers blind to the experimental condition separately counted the number of marbles buried (defined as > 2/3rds covered in bedding). In the event of a discrepancy, the researchers examined the marble together and came to a consensus. The sole measure from this test was the number of marbles buried by the mice during the 30-minute period. As in Hypotheses 4 and 6, a second positive control was employed to validate this measure. Mice (N = 10/group) were given a single i.p. injection of either 2 g/kg ETOH or saline 5 minutes before introduction to the MBT chamber.

3.4.7: H7: Toluene re-exposure following toluene abstinence

To assess whether re-exposure to toluene vapor would ameliorate the symptoms of toluene abstinence, toluene-abstinent mice were placed back in the dynamic exposure system and re-exposed to 5,000 ppm toluene vapor for 30 minutes. To investigate the impact of toluene exposure on PTZ-induced seizures (per Hypothesis 2 and 3), two groups of mice, after 24 hours of either Air or 5,000 ppm and 3 hours of abstinence were exposed to toluene vapor for 30 minutes. Immediately following exposure, mice were treated with 42 mg/kg PTZ as previously described (Hypothesis 3). To examine the effect of toluene re-exposure on anxiety behaviors, as well as the

impact of exposing animals to the novel static exposure system, a single cohort of mice received a 30-minute re-exposure to toluene vapor following the 24-hour abstinence protocol described in Hypothesis 4 (24 hours of 5,000 ppm toluene followed by 24 hours abstinence; $N = 10$). A second cohort served as the control animals (24 hours of air followed by 24 hours abstinence; $N = 10$) and also received 5,000 ppm toluene. Following the 30-minute re-exposure, these mice were immediately tested in the EPM following the protocol detailed in Hypothesis 4.

3.5: Data Analysis

The 72-hour weight change data was analyzed with a 3 x 2 Mixed design ANOVA utilizing SPSS (Version 23, IBM). All other statistical tests were performed utilizing Graph Pad Prism 6 (GraphPad Software). Seizure data was analyzed using Chi Square tests of goodness of fit. Anxiety-like behavior was analyzed using t-tests for independence or One-Way ANOVAs. Significance was set at alpha = 0.05.

CHAPTER 4: RESULTS

4.1: 24-hour Toluene Exposure: Concentration Stability

Toluene concentrations within the exposure chamber reached maximum concentration within ~ 8 minutes. Toluene concentrations were monitored hourly for the first 12 hours and any fluctuations from the desired concentration during this time were corrected through manual adjustment of the flow regulators. The entire 24-hour period was recorded and examined to ensure that the concentration was within ±5% of the target concentration for the duration of exposure period. An example 24-hour exposure is shown in *Figure 3.* The air-control chamber remained at zero (i.e., no toluene) for the duration of exposure.

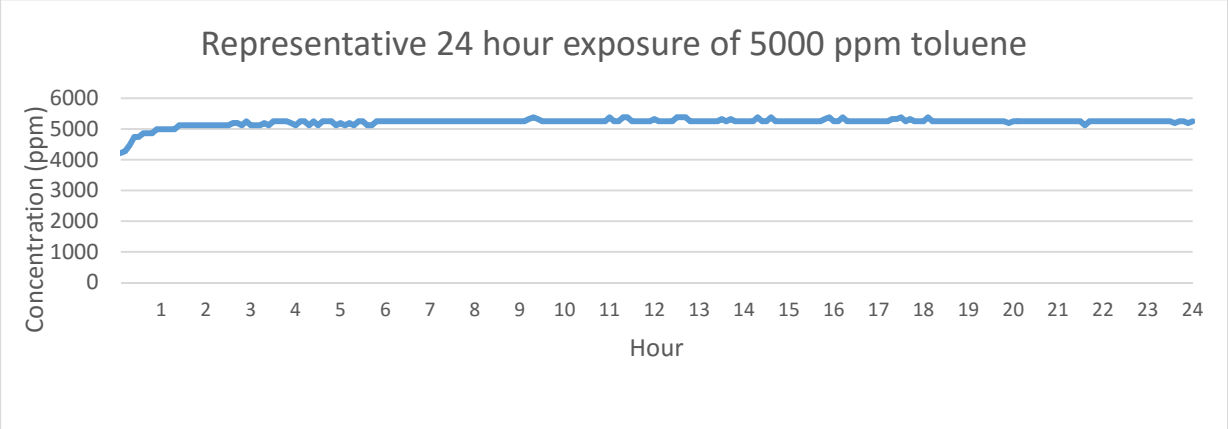

Figure 3: An example 24-hour plot of toluene vapor concentration.

4.2: Weight Change following 24-hour continuous toluene exposure.

As shown in Figure 4, animals exposed to 24 hours of a continuous concentration of 5,000 ppm toluene weighed significantly less than air controls, t (46) = -2.98, $p < .05$. Although food and water levels were not directly measured, it is likely that the weight loss observed in the toluene animals was due to a reduction in water and food consumption during the toluene exposure period.

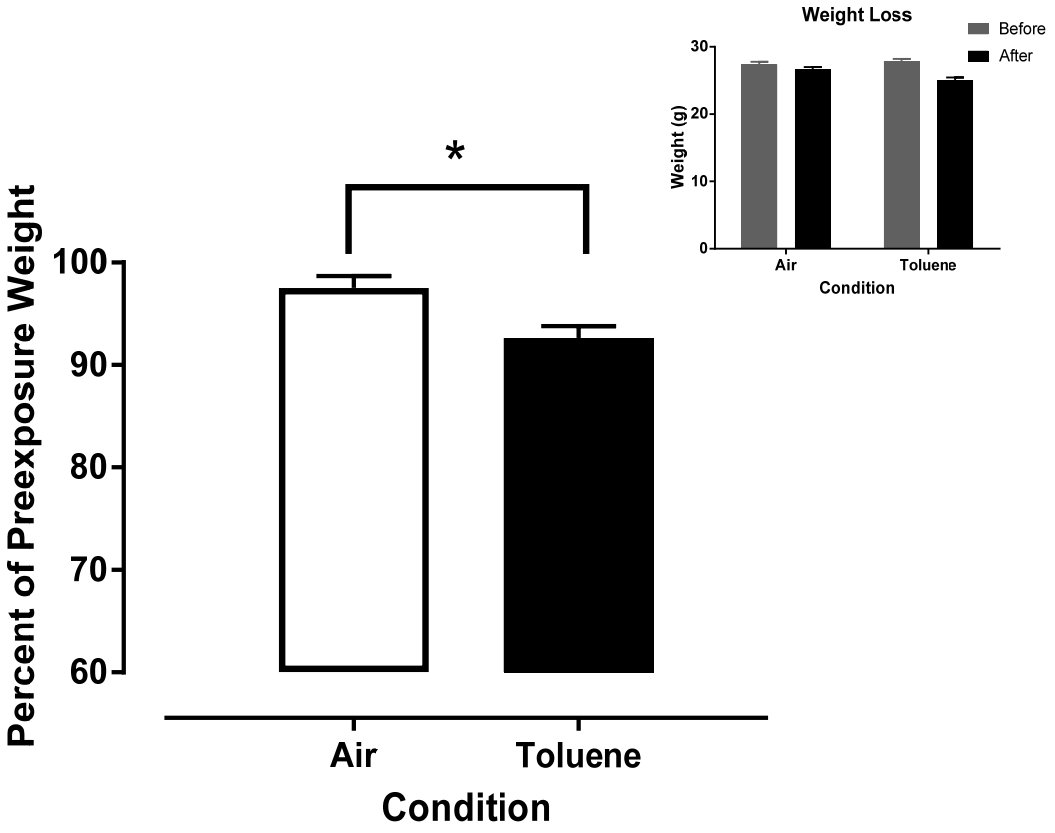

Figure 4: Shown are the mean weights (as a percent of baseline ± SEM) following a 24-hour exposure to 5,000 ppm toluene vapor in mice tested in Hypotheses 1-3. Mice exposed to toluene weighed significantly less than air control mice, t (46) = -2.98, $p < .05$. Inset represents mean animal weights (± SEM) before and after 24-hour toluene exposure.

4.3: Recovery from weight loss due to toluene exposure

As seen in Figure 5, mice that were exposed to toluene for 24 hours and then allowed to recover for up to 72 hours experienced similar weight loss to those in the previous experiment who were weighed immediately after a 24-hour exposure (Figure 4). There was a significant between-subjects effect of toluene exposure, F (1, 52) = 101.34, $p < .001$, with toluene-exposed mice weighing less than the air-exposed mice. Both toluene exposed and air control animals displayed significant weight gain during the 72-hour recovery period, F (2, 104) = 101.10, $p < .001$. There was a significant treatment condition X time interaction, F (1, 104) = 13.29, $p < .001$, indicating

that toluene exposed animals gained weight significantly faster than air control animals (see Figure 5). Seventy-two hours post toluene exposure, mice that had been previously exposed to toluene had recovered to 98% of their pre-exposure weight, while air-control mice had reached 101.5% of their base weight.

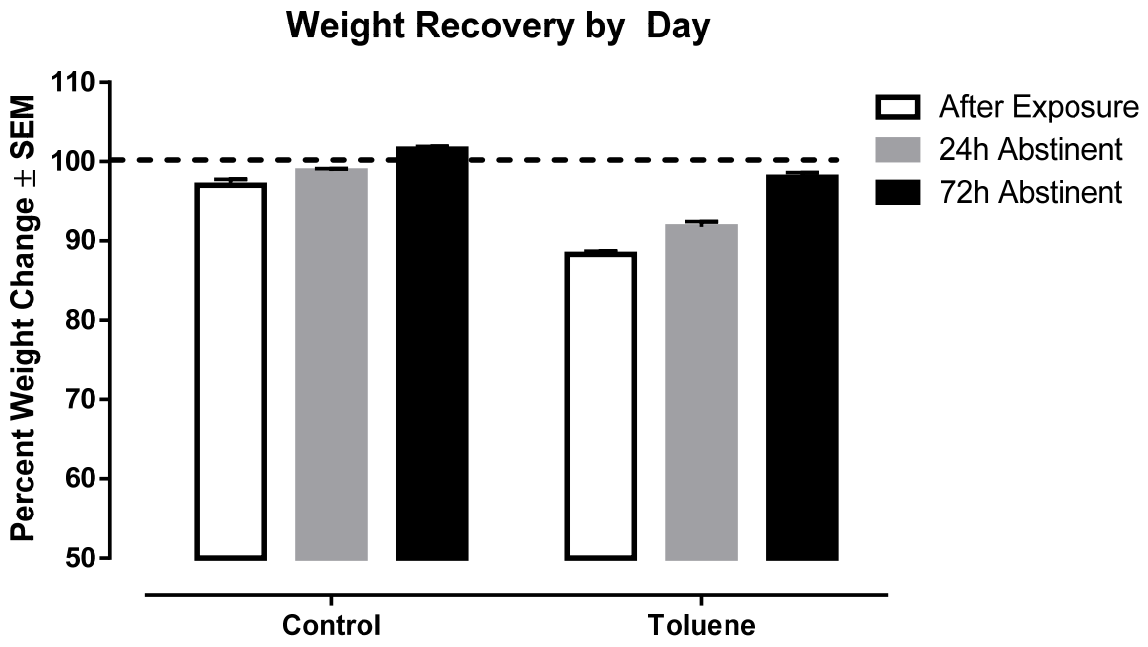

Figure 5: Shown are daily changes in weight for mice exposed to either air (left side) or 5000 ppm toluene (right side) during the 72-hour recovery period. Data are represented as mean percent of baseline weight ± SEM. Note that the Y axis is offset to better highlight differences over time.

4.4: H1: Handling-induced seizure activity following acute massed toluene exposure

Similar to what has been previously reported with 1,1,1-TCE (Evans and Balster, 1993), seizure onset was relatively quick following cessation of toluene exposure. Approximately 71% of mice in the toluene exposure condition (total n = 7) displayed signs of clonic seizure (i.e., mild tremor, etc.) activity one hour following removal from the exposure chamber. The percent of animals displaying seizure activity (100%) peaked at four hours post toluene exposure before

decreasing in subsequent hours. Twenty-four hours after toluene exposure, the majority of mice (N = 5 of 7) were no longer having handling-induced seizures following the tail-twist test and all animals had fully recovered by 48 hours post toluene exposure. No mice experienced the more severe tonic seizures during this experiment nor were there any fatalities. It should be noted that none of the air control animals displayed seizure activity at any time. No statistics were performed, as the variance for the air control animals was zero. This information is summarized on *Figures 6 and 7*.

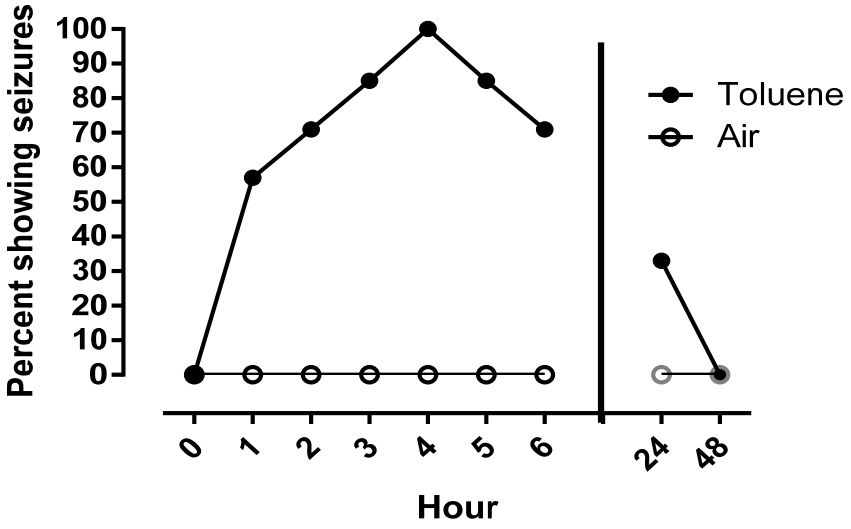

Figure 6: The effect of 24-hour exposure to 5,000 ppm toluene on handling-induced seizures. Data are represented as the percentage of animals in each group displaying a behavioral sign of seizure activity following handling at each time point following toluene exposure (N = 8 for air controls, N = 7 for toluene animals). All seizures observed were clonic (consisting of mild to severe tremors). No mice experienced severe clonic convulsions or "wet dog" shakes. Data represented as % of total due to unequal between-groups sample size. There are no error bars as this data is based on frequency information.

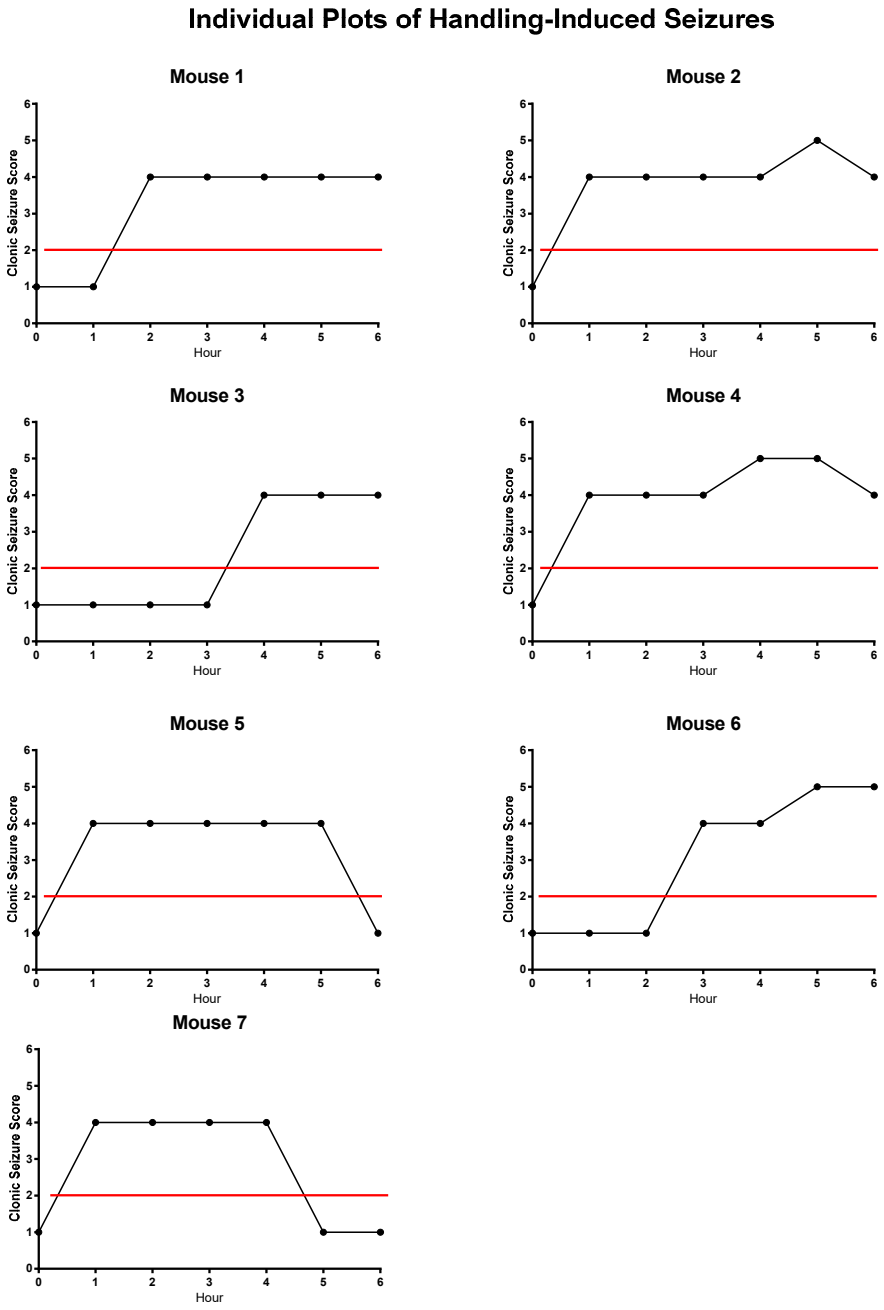

Figure 7: Shown are individual mouse plots by hour of handling-induced seizures following 24-hour exposure to 5,000 ppm toluene vapor. The horizontal red line signifies the threshold for significant symptoms (i.e., scores above the red line are considered "seizure symptoms").

4.5: H2: The effect of abstinence following 24-hour exposure to 5,000 ppm toluene vapor on PTZ-induced seizure activity.

While toluene abstinence did not increase the relative frequency of animals responding to PTZ (N = 18 / group) as compared to air controls (p = 0.12), it did significantly increase the categorical severity of seizures experienced by animals previously exposed to toluene, χ^2 (2) = 6.47, $p < 0.05$. Put another way, when toluene-abstinent animals had seizures, these seizures tended to be more severe as compared to air-control animals. This increased severity resulted in both a higher proportion of tonic symptoms and categorically more tonic and clonic symptoms compared to air controls (see *Figures 8, and 9 and Table 2*). Particularly, the toluene exposed animals displayed a higher frequency of clonic convulsions and myoclonic jerk movements. No mice experienced tonic-only seizures nor were there any lethalities.

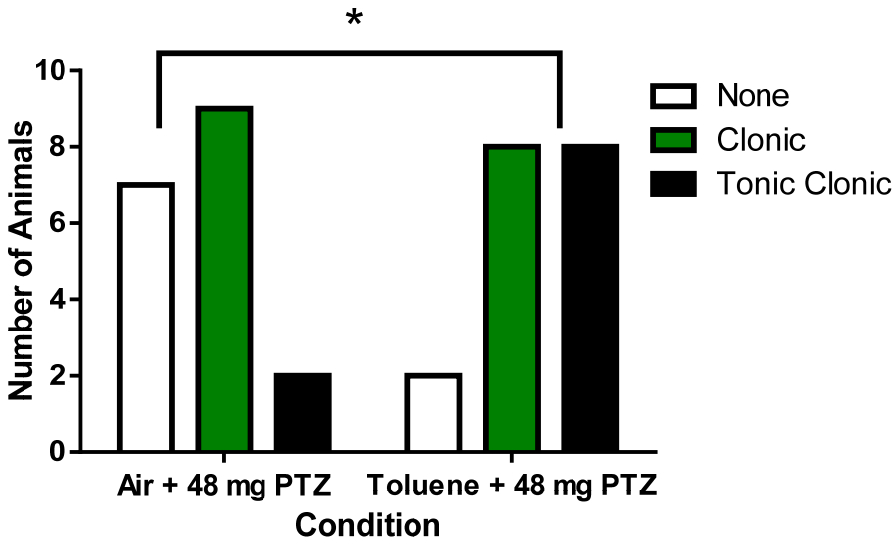

Figure 8: Shown were the number of mice experiencing seizures after an injection of 48 mg/kg PTZ following a 3-hour acute exposure of air (left side) or toluene (right side). Data are represented as the number of animals scored in each category (N=18). *χ^2 (2) = 6.47, $p < 0.05$. No error bars are present as this is frequency based data.

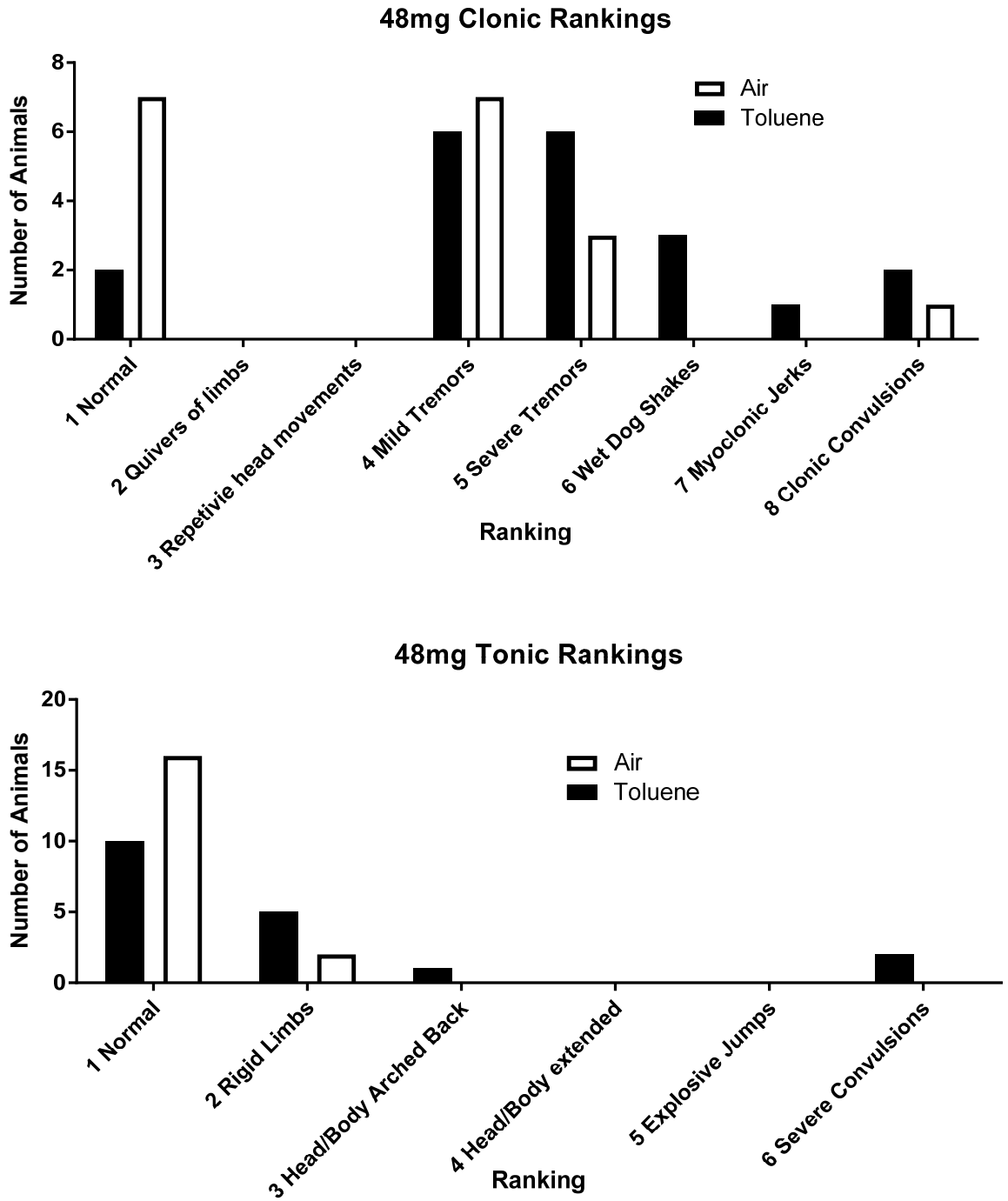

Figure 9: Shown are the number of animals displaying specific symptoms of clonic (above) and tonic (below) seizures after administration of 48mg/kg PTZ 3 hours following 24-hour exposure to either air or 5,000 ppm toluene vapor (N = 18/group).

4.6: Hypotheses 3 and 8: The effect of abstinence (with or without a 30-minute re-exposure to toluene) following 24-hour exposure to 5,000 ppm toluene vapor on 42mg/kg PTZ-induced seizure activity

Observation of animals given an i.p. injection of 42 mg/kg PTZ 3 hours after a 24-hour exposure to 5,000 ppm of toluene vapor, revealed qualitatively more severe seizures than mice that had only been exposed to 24 hours of air, χ^2 (2) = 7.57, $p < .05$. This suggests that toluene-abstinent animals were more sensitive to a sub-threshold concentration of PTZ than air-control animals. Re-exposure to 5,000 ppm toluene vapor for 30 minutes produced qualitatively less severe seizures as compared to mice that were not re-exposed to toluene, χ^2 (2) = 6.00, $p <.05$, suggesting that toluene re-exposure reduces the influence of toluene abstinence on PTZ. Air-control mice and air-control mice with a 30-minute toluene exposure did not significantly differ in seizure severity. These results are summarized in *Figures 10, 11, and 12*.

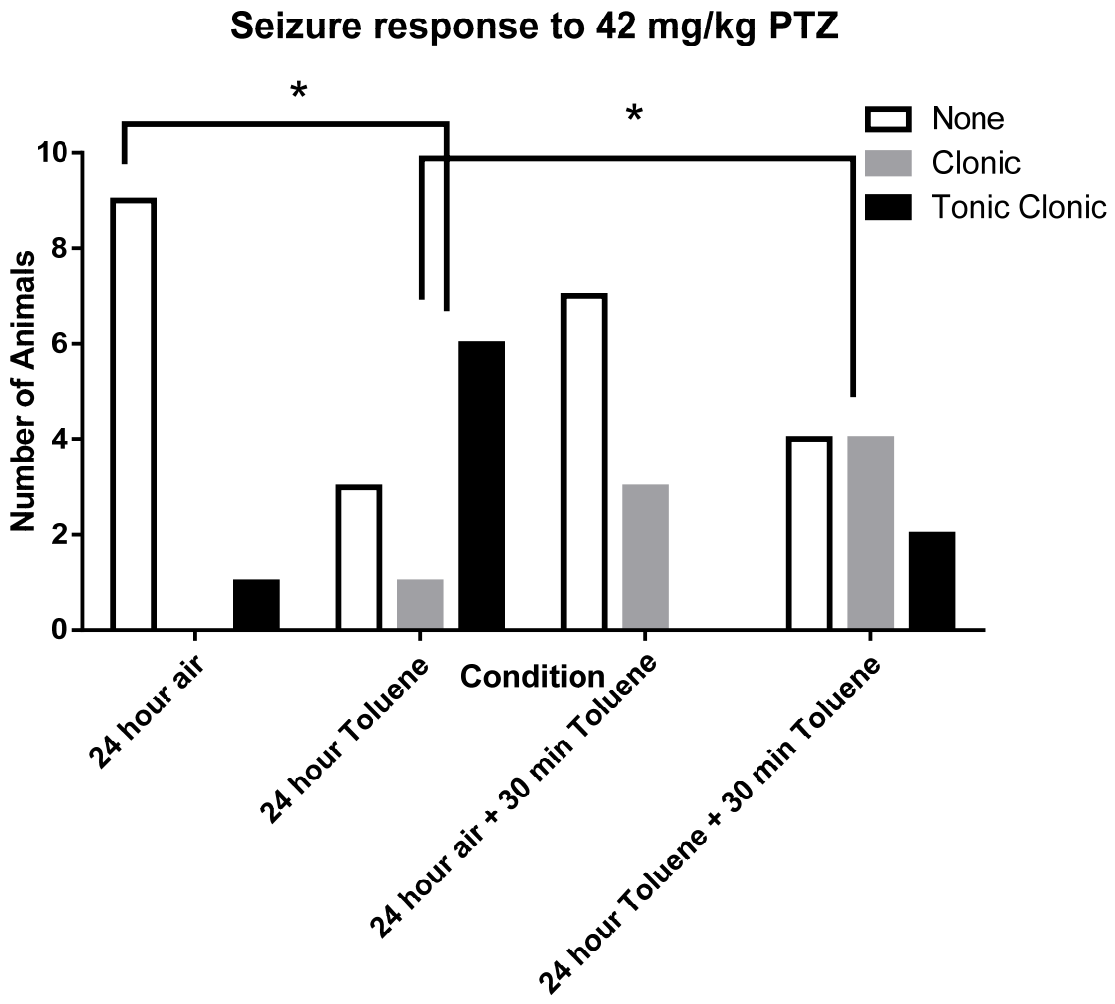

Figure 10: Shown are the number of seizures induced by 42 mg/kg PTZ administered 3 hours following a 24-hour exposure to either air or 5,000 ppm toluene with or without a 30-minute re-exposure to 5,000 ppm toluene. Data are shown as the number of animals scored in each category. There are no error bars as this data is frequency based.

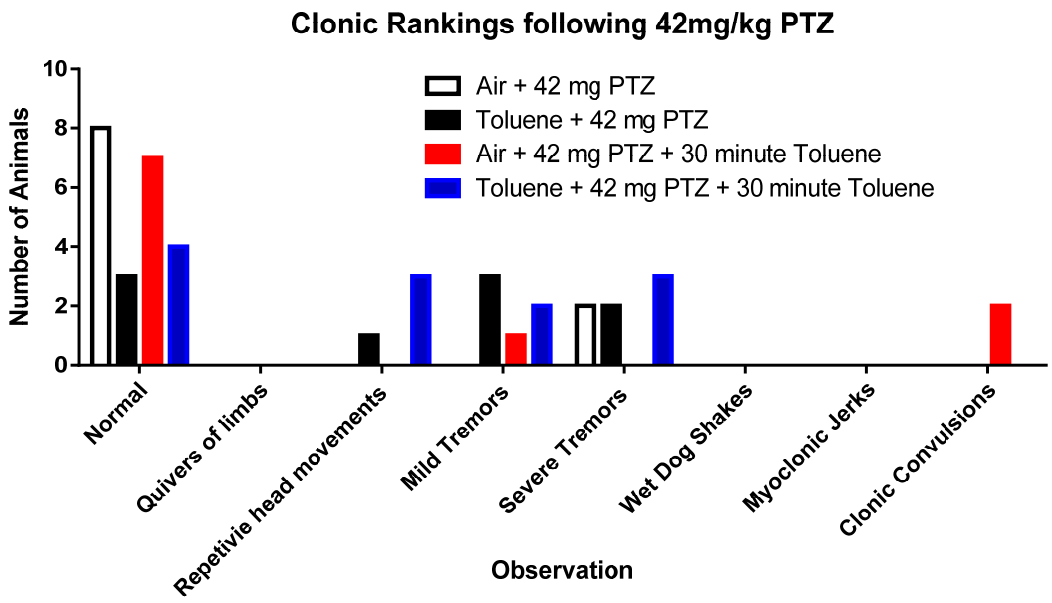

Figure 11: Shown are number of animals displaying clonic seizure activity in response to administration of 42mg/kg PTZ 3 hours after a 24-hour exposure to either air or 5,000 ppm toluene vapor with or without a 30-minute re-exposure to 5,000 ppm toluene (N = 10/group).

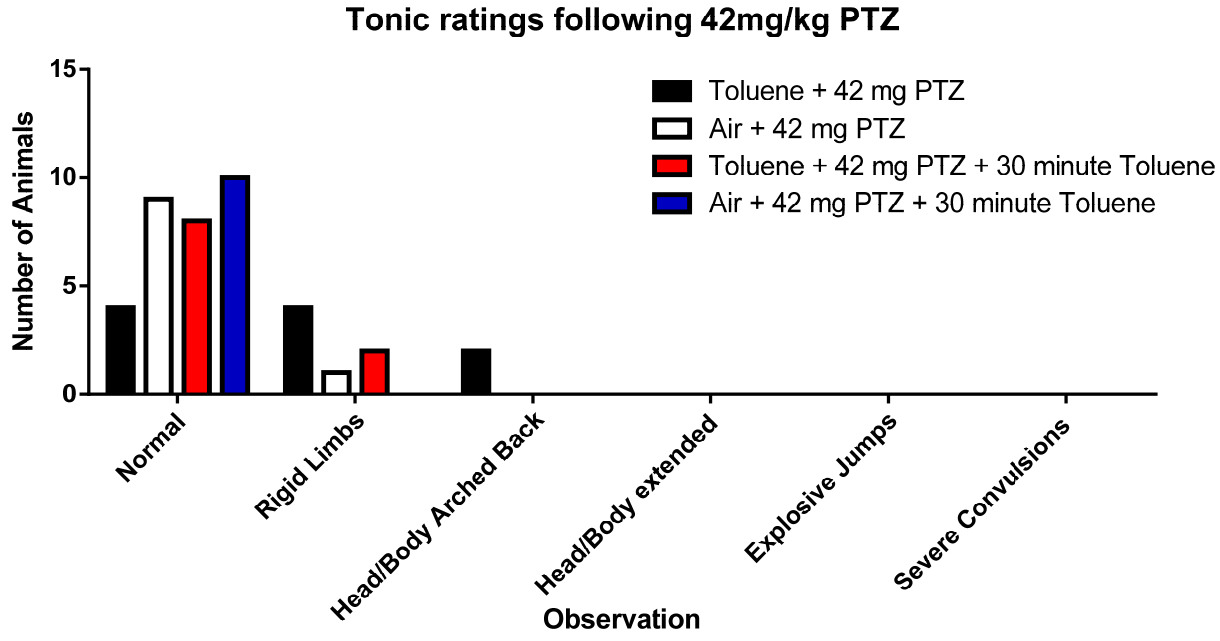

Figure 12: Shown are the number of animals displaying tonic seizure activity in response to administration of 42mg/kg PTZ 3 hours after a 24-hour exposure to either air or 5,000 ppm toluene vapor with or without a 30-minute re-exposure to 5,000 ppm toluene (N = 10/group).

4.7: Hypothesis 4: Elevated Plus Maze (EPM) test for anxiety-like behavior during toluene abstinence

Prior to testing toluene-exposed animals in the EPM, a positive control was conducted using ETOH to ensure the validity of the behavioral test. As seen in the left panel of Figure 14, mice given a dose of 2 g/kg ETOH spent significantly more time on the open arms of the EPM as compared to saline control mice, t (15) = 5.91, $p < .001$. This increase in open arm time was not accompanied by a significant increase in distance traveled or number of open arm entries, t (15) = 0.46, $p = 0.65$, t (15) = 0.97, $p = 0.35$.

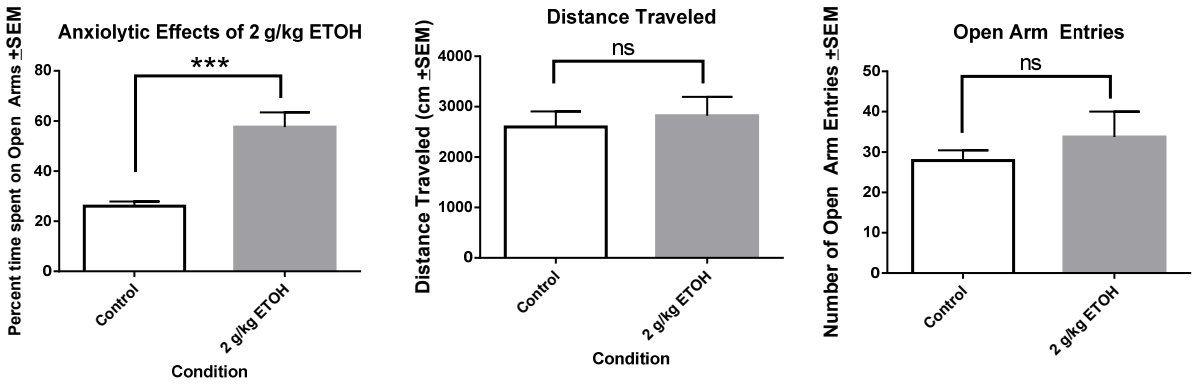

Figure 13: Shown is the effect of acute 2 g/kg i.p. ETOH injection on performance on the EPM task. The left panel depicts the percent of the 5-minute trial the animal spent on the open arms. The middle panel shows the total distance traveled during the trial. The right panel displays the number of entries into the open arms of the maze. Data for all panels are represented as mean ± SEM.

Following verification of the EPM with ETOH, additional mice were evaluated on the EPM 24 or 72 hours after being exposed to 24 hours of toluene (5000 ppm). As seen in Figure 14, mice abstinent for 24 hours displayed a significant decrease in open arm exploration as compared to 24 hour abstinent air controls, t (17) 2.65, $p < .05$. These same mice did not differ from air controls in terms of distance traveled, t (17) = 0.42, $p = 0.67$; or number of open arm entries, t (17) = 1.09, $p = 0.28$ (Figure 15). Mice abstinent for 72 hours did not differ from controls in terms of time spent

on the open arms, t (17) = 0.04, p = 0.96; distance traveled, t (17) = 0.73, p = 0.47, or number of open arm entries, t (17) = 1.67, p = 0.11.

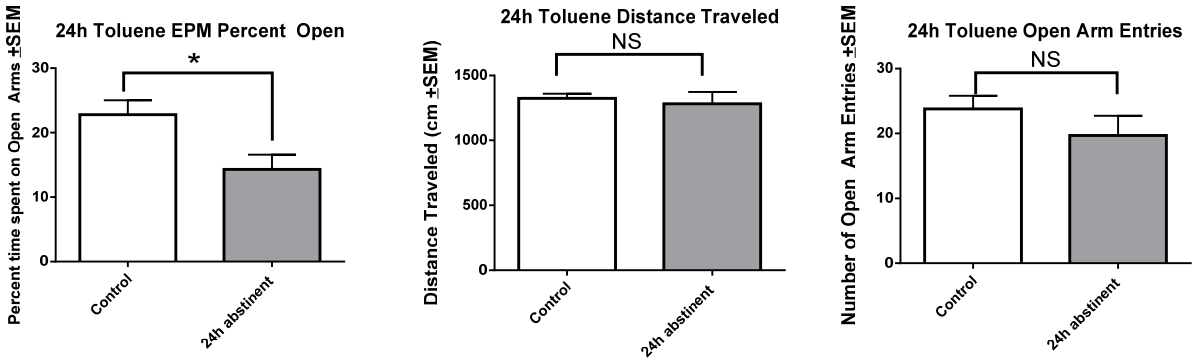

Figure 14: Shown are the effects of 24-hour abstinence following 24-hour exposure to 5,000 ppm toluene on anxiety-like behavior in the EPM. Shown on the left is the percent of total time spent on the open arm. The middle panel depicts the total distance traveled in centimeters during the maze trial. The right panel shows the number of open arm entrances made during the trial. All data are represented as mean ± SEM.

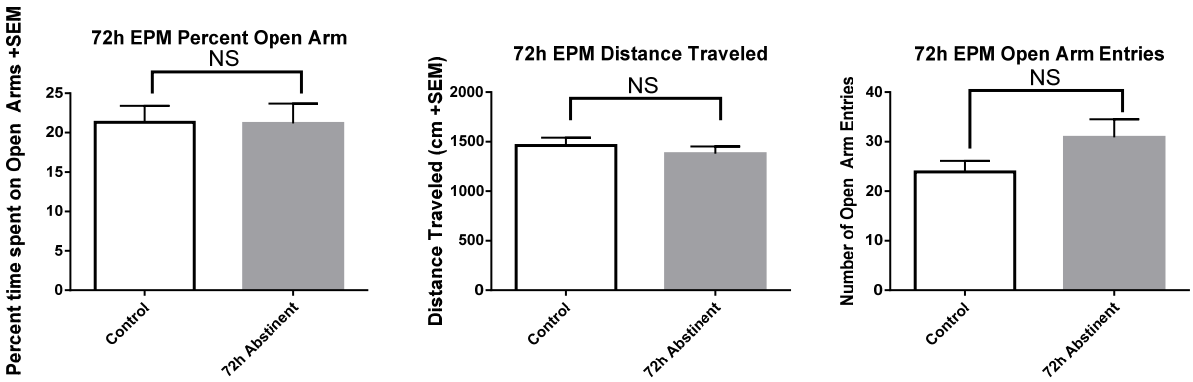

Figure 15: Shown are the effects of 72-hour abstinence following 24-hour exposure to 5,000 ppm toluene on anxiety-like behavior in the EPM. Shown on the left is the percent of total time spent on the open arm. The middle panel depicts the total distance traveled in centimeters during the maze trial. The right panel shows the number of open arm entrances made during the trial. All data are represented as mean ± SEM.

An acute test with 30 minutes of 5,000 ppm toluene vapor was also conducted and compared to a 24-hour abstinent group that had been re-exposed to toluene vapor for 30 minutes. As seen in Figure 16, examination of the percent of time spent on the open arms revealed a

significant omnibus effect of treatment condition, F (3, 35) = 7.33, $p < .001$, with the three toluene exposure conditions having no significant differences from each other. Post-hoc tests revealed that the air-only control showed significantly lower percent-time on the open arm as compared to the 24 hour "abstinent" air + 30 min toluene ($p < .001$), the 30-minute acute toluene ($p < .05$), and the 24-hour abstinent toluene + 30 min toluene re-exposure ($p < .001$). In terms of distance traveled during testing, there was again a significant omnibus effect, F (3, 35) = 8.85, $p < .001$. Post-hoc test reveal that the 24-hour "abstinent" air control group + 30-minute toluene exposure was significantly less active than the air only control ($p < .001$). For the number of open arm entries, there was no significant effect of toluene exposure, F (3,35) = 1.60, $p = 0.20$. These results are summarized in Figure 16.

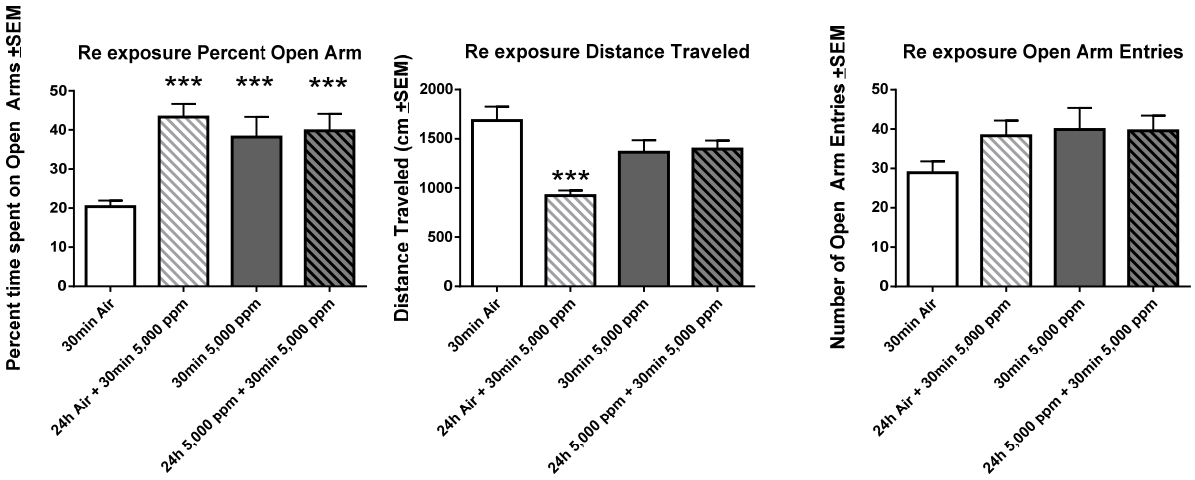

Figure 16: Shown are the effects of various toluene exposure times on abstinence-induced anxiety-like behavior in mice. Shown on the left is the percent of total time spent on the open arm. The middle panel depicts the total distance traveled in centimeters during the maze trial. The right panel shows the number of open arm entrances made during the trial. All data are represented as mean ± SEM.

4.8: Light Dark Box for anxiety-like behavior

To validate the Light/Dark Box, a positive control of acute 2 g/kg ETOH was used. As seen in Figure 17, a single injection of 2g/kg of ETOH significantly increased the percent of time

spent in the lit chamber, t (14) = 2.61, $p < .05$, but did not affect the latency to first enter the dark chamber, t (16) = 0.75, $p = 0.46$.

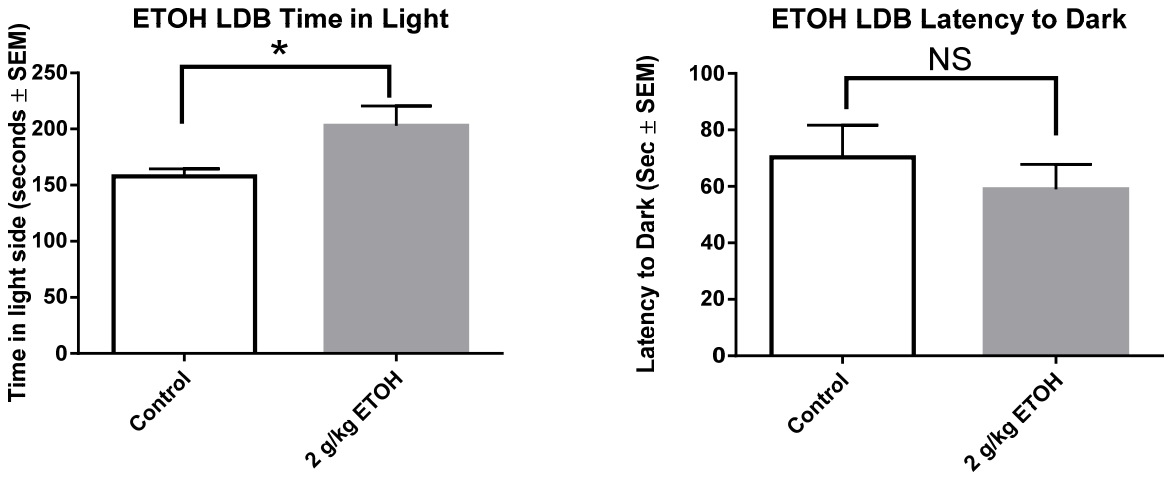

Figure 17: Shown are the effects of a single injection of ETOH (2g/kg) on anxiety-like behavior as measured by the Light/Dark Box (LDB) test. Shown on the left is the total time in seconds spent on the lit side of the LDB apparatus. On the right is the latency in seconds before the animal first crossed into the dark chamber. Data here are represented as mean ± SEM.

A thirty minute exposure of 5,000 ppm toluene did not significantly affect time spent in the lit compartment, t (17) = 1.41, $p = 0.17$. Toluene-exposed animals were significantly less active in the lit compartment than air controls, t (17) = 2.34, $p < .05$, had significantly less dark-zone entrances than air controls, t (16) = 5.22, $p < .001$, and had a significantly higher latency to first enter the dark zone, t (16) = 2.55, $p < .05$, as compared to air controls (Figure 18).

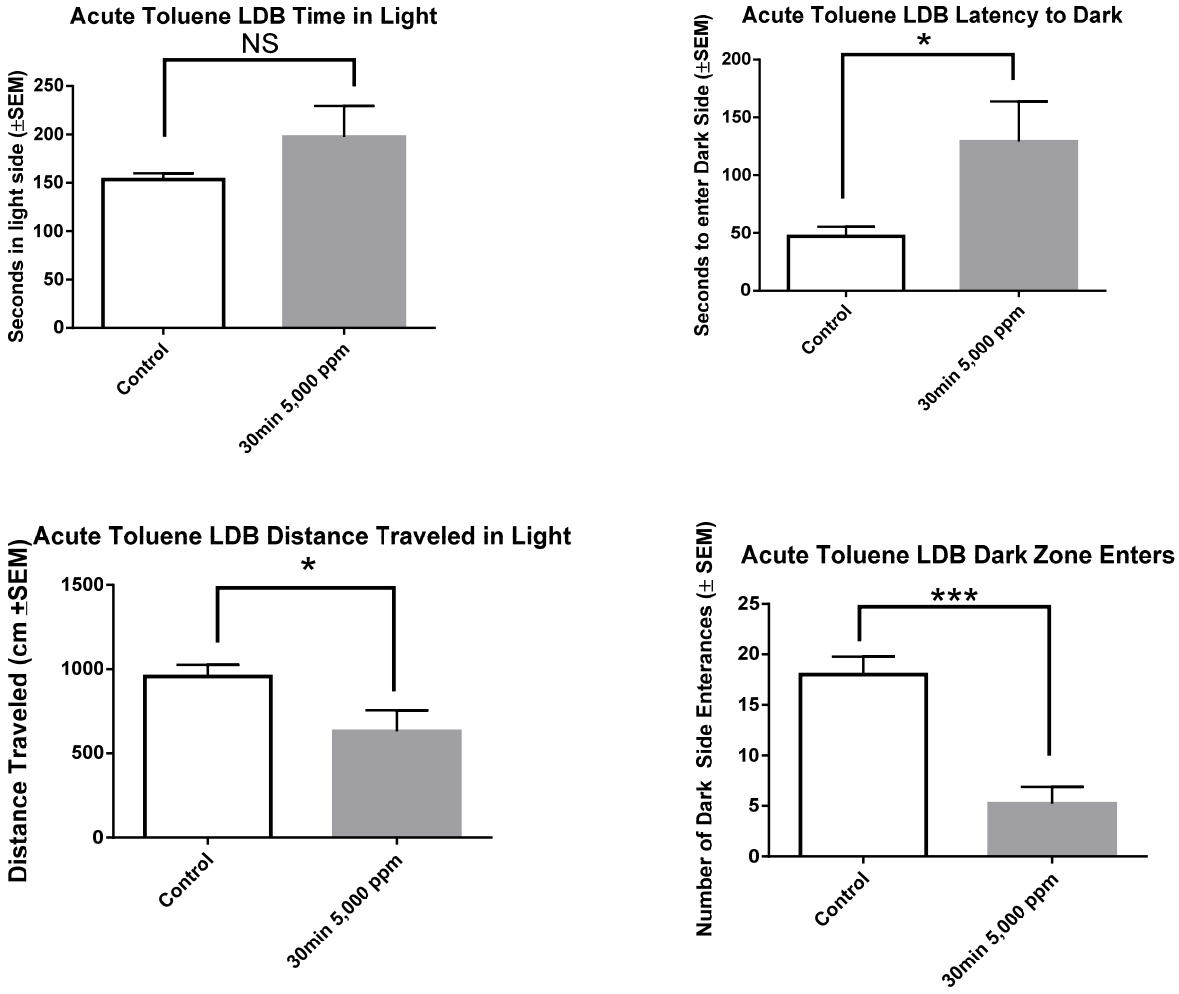

Figure 18: The effect of acute toluene exposure on anxiety-like behavior in the Light Dark Box (LDB). The upper left panel represents the total time in seconds spent in the lit section of the LDB apparatus. The upper right box represents the latency to first entering the darkened chamber. The bottom left box depicts the total distance traveled in the lit compartment during the testing session, while the lower right box depicts the number of dark zone transitions during exposure. All data here are represented as mean ± SEM.

As seen in Figure 19, twenty-four hours after a 24-hour exposure of toluene (5,000 ppm), mice displayed a significant reduction of time spent in the light chamber as compared to air controls, t (18) = 2.46, $p < .05$. Toluene abstinence also significantly reduced the latency to first

entering the dark chamber, t (17) = 4.32, $p < .001$, but it did not impact distance traveled, t (18) = 1.80, $p = .08$, nor did it affect the number of dark zone entrances, t (18) = 1.44, $p = 0.16$.

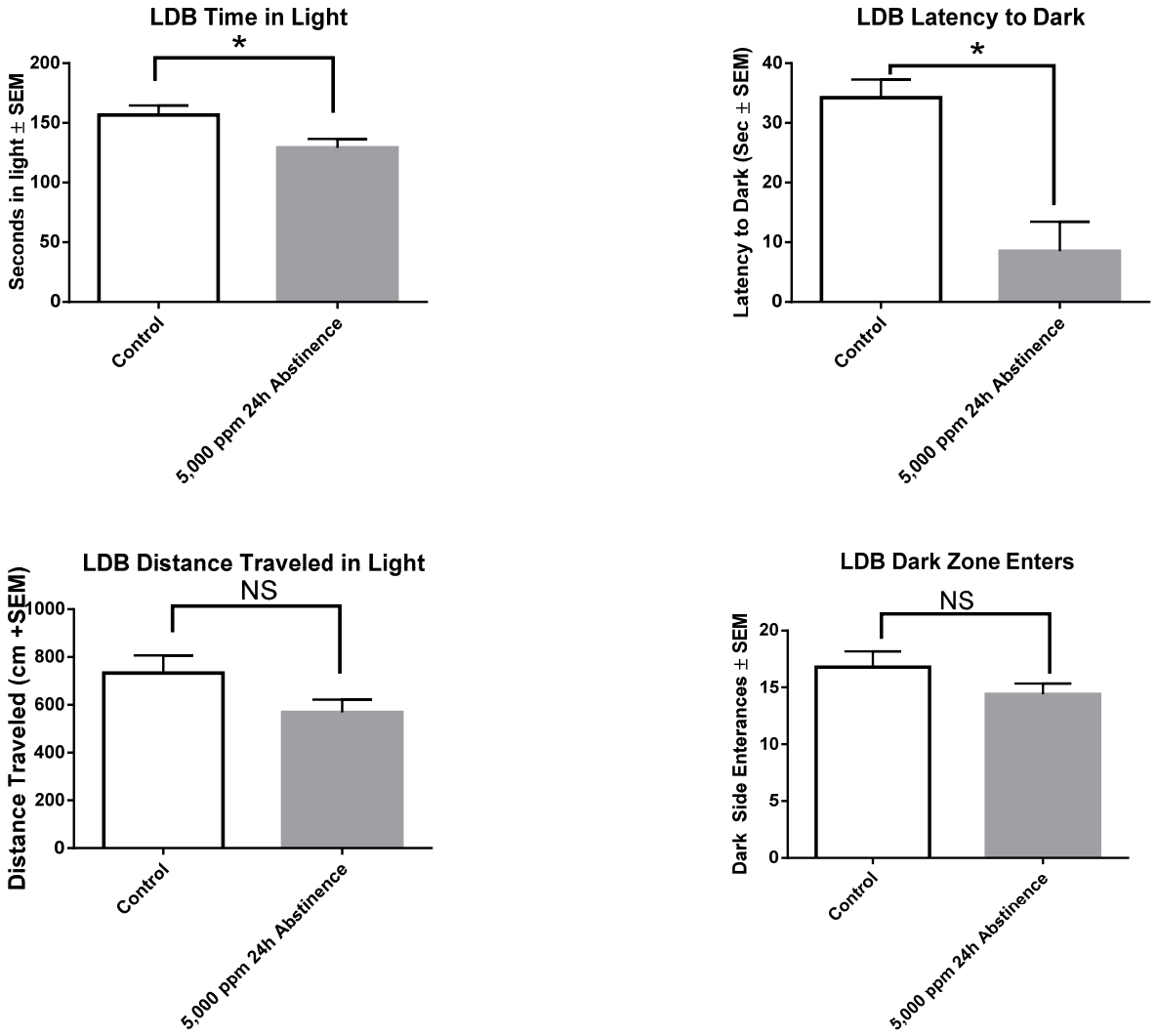

Figure 19: Shown are the effects of 24-hour toluene exposure and 24-hour toluene abstinence on anxiety-performance in the LDB test. The upper left panel represents the total time in seconds spent in the lit section of the LDB apparatus. The upper right box represents the latency to first entering the darkened chamber. The bottom left box depicts the total distance traveled in the lit compartment during the testing session, while the lower right box depicts the number of dark zone transitions during exposure. All data here are represented as mean ± SEM.

Mice tested 72 hours after a 24-hour exposure to toluene (5,000 ppm) did not spend more time on the light side of the chamber as compared to air controls, t (14) = 1.70, p = 0.11. Likewise, latency to first entering the dark side of the chamber was unaffected following the 72-hour abstinence, t (14) = 1.50, p = 0.15. However, these same animals did show significantly fewer dark-zone transitions as compared to controls, t (14) = 3.09, p < .01, though distance traveled was unimpaired, t (14) = 1.83, p = 0.083 (see Figure 20).

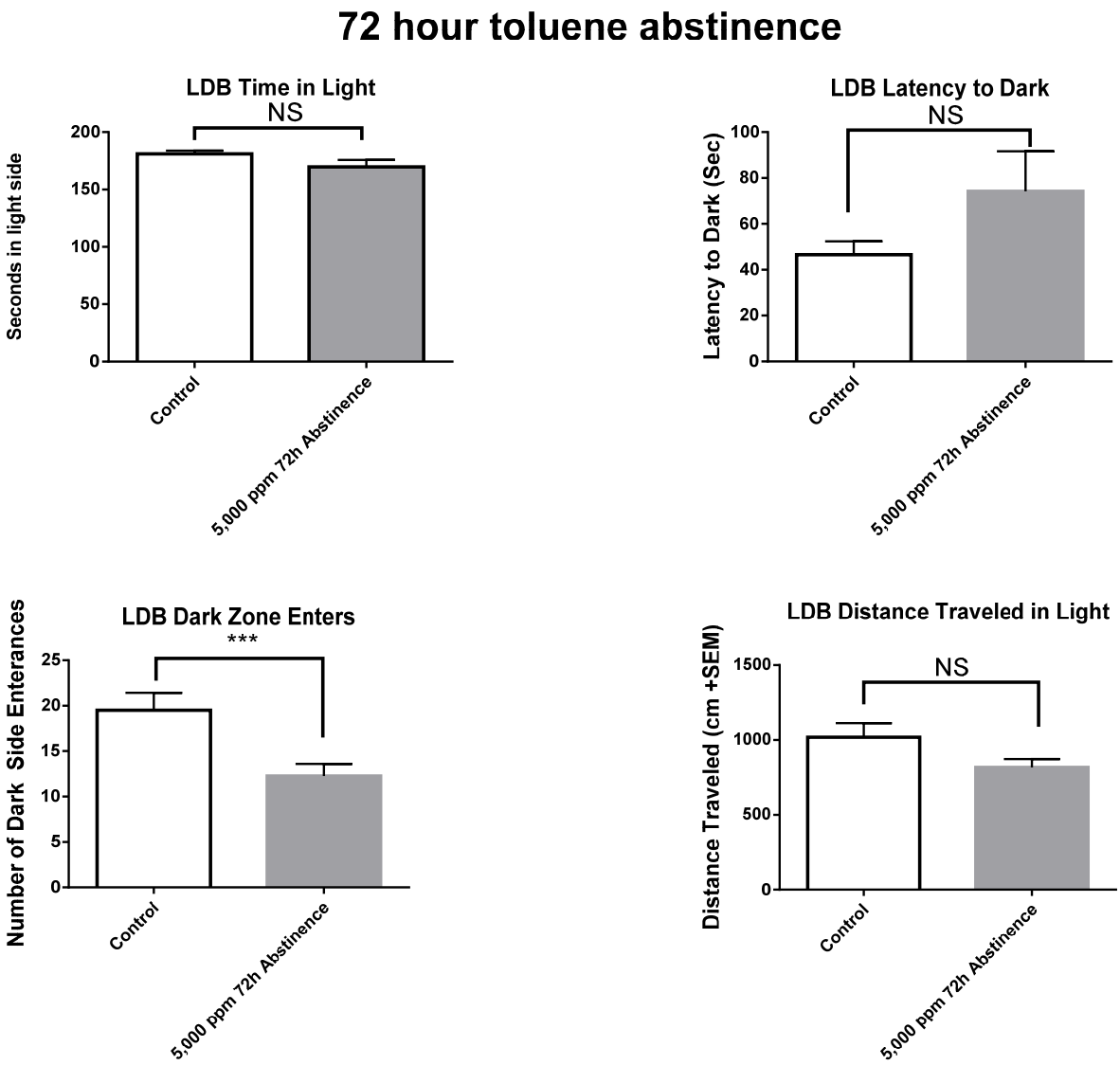

Figure 20: Shown are the effects of 24-hour exposure to 5,000 ppm toluene (or air control) after a 72-hour abstinence period on anxiety-like behavior in the LDB task. The upper left panel represents the total time in seconds spent in the lit section of the LDB apparatus. The upper right box represents the latency to first entering the darkened chamber. The bottom left box depicts the total distance traveled in the lit compartment during the testing session, while the lower right box depicts the number of dark zone transitions during exposure. All data here are represented as mean ± SEM.

4.9: Open Field Test

For the Open Field Test, 30 minutes of 5,000 ppm toluene exposure did not significantly increase time spent in the center section, t (15) = 1.33, p = 0.22. However, 24 hours after toluene exposure, mice spent less time in the center section as compared to controls, t (14) = 2.40, $p < .05$. There was no significant difference in center square preference for animals in the 72-hour abstinence experiment, t (16) = 0.75, p = 0.46. These results are summarized in Figure 21.

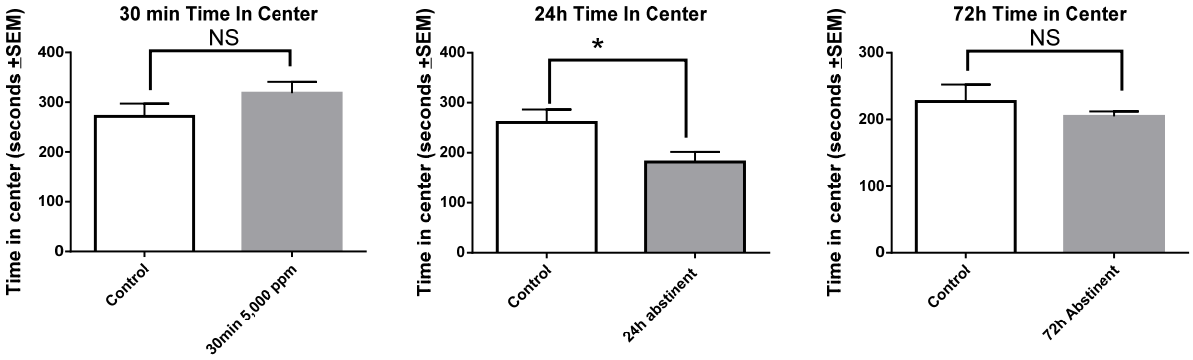

Figure 21: Anxiety-like behavior as measured by the Open Field Test. The left panel depicts the effect of 30-minute acute exposure to 5,000 ppm toluene on anxiety-like behavior as measured by time spent in the center of the arena. The middle panel shows effect of 24-hour exposure to 5,000 ppm toluene vapor after a 24-hour abstinence period on anxiety-like behavior as measured by time spent in the center of the arena. The right panel displays effect of 24-hour exposure to 5,000 ppm toluene vapor after a 72-hour abstinence period on anxiety-like behavior as measured by time spent in the center of the arena.

As shown in Figure 22, an acute 30-minute exposure to toluene did not alter locomotor activity (distance traveled) during the OFT, t (17) = 1.77, p = 0.093. Likewise, 24 hours of

abstinence following 24-hour toluene exposure did not affect total distance traveled during OFT testing, t (14) = 0.78, p = 0.44. Finally, 72-hour abstinence following 24-hour toluene exposure did not affect distance traveled in the OFT, t (16) = 0.87, p = 0.397.

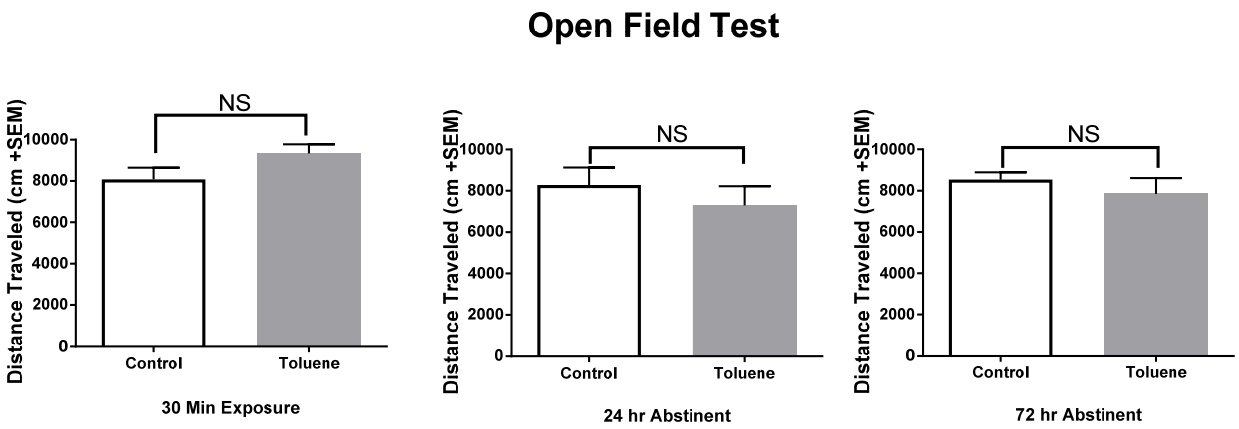

Figure 22: Shown are the effects of a 30 min exposure to 5000 ppm of toluene (left panel) and 24-hours after a 24-hour exposure to 5000 ppm toluene (middle panel) and 72-hours after a 24-hour exposure to 5000 ppm toluene (right panel) on distance traveled during the Open Field Test.

4.10: Marble Burying Task for anxiety-like behavior

Before assessing the effect of toluene and toluene abstinence in the marble burying task, a positive control of acute 2g/kg ETOH was tested. Acute ETOH administration significantly decreased the number of marbles buried during the 30-minute test period, t (18) = 9.97, p < .001 (Figure 23).

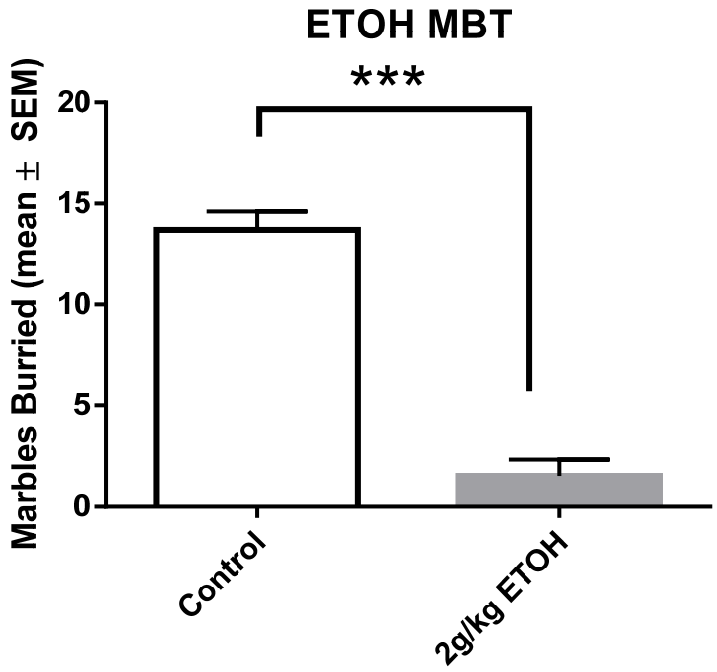

Figure 23: Shown are the effects of a 2 g/kg i.p. injection of ETOH or saline on the number of marbles buried during a 30-minute observation period.

As seen in Figure 24, an acute 30-minute exposure of 5,000 ppm toluene significantly decreased the number of marbles buried as compared to air controls, $t\,(17) = 3.72$, $p < .01$. Animals exposed to 24 hours of 5,000 ppm toluene and allowed to recover for 24 hours buried significantly more marbles than air control animals, $t\,(17) = 3.20$, $p < .01$. Animals allowed to recover for 72 hours following toluene exposure did not differ from control animals in terms of the number of marbles buried, $t\,(16) = 1.00$, $p = 0.33$. These results are summarized in Figure 24.

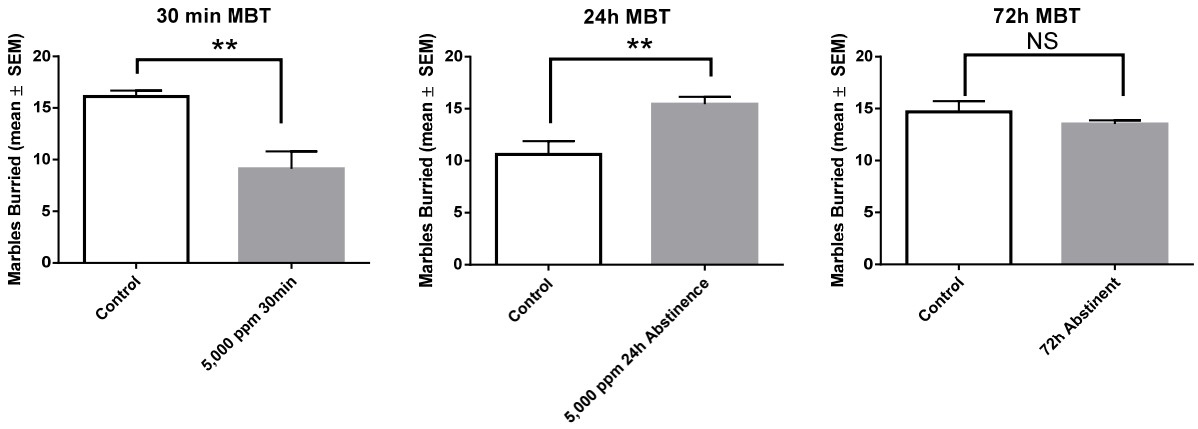

Figure 24: The effect of toluene exposure and abstinence on the number of marbles buried during the MBT. Shown on the left are the number of marbles buried following a 30 minute exposure to either air or 5,000 ppm toluene. The middle panel depicts the number of marbles buried by animals 24 hours abstinent following a 24 hour exposure to either 5,000 ppm toluene or air control. The right panel depicts the number of marbles buried by animals 72 hours abstinent following a 24 hour exposure to either 5,000 ppm toluene or air. All data here are represented as mean ± SEM.

CHAPTER 5: DISCUSSION

This project was designed to examine in a preclinical animal model two commonly reported symptoms of inhalant withdrawal (seizures and anxiety) using a novel form of our previously established toluene exposure paradigm. The present experiments represent the first pre-clinical evidence of a toluene-specific abstinence syndrome, and confirm the hypothesis first put forward by Perron and colleagues (Perron et al., 2009) that physical dependence to an inhalant can result in withdrawal symptoms (other than craving) occurring following inhalant exposure. We were able to reliably and repeatedly elicit behaviors indicative of anxiety across four distinct assays, as well as, increase handling-induced seizure severity across a 24-hour period following a single 24-hour exposure to 5,000 ppm toluene vapor. Further confirmation of withdrawal-related hyper excitability was corroborated by challenging mice during the peak period of toluene withdrawal and the subsequent demonstration of a significant increase in seizure activity following treatment with PTZ. At both the lower (42mg/kg) and higher (48mg/kg) concentrations, PTZ significantly elevated seizure severity in the toluene abstinent groups as compared to air controls. Moreover, we were able to demonstrate that both anxiety-like and seizure behaviors were transient in nature and could be ameliorated by re-administering toluene vapor. Our assays were highly consistent both internally and with the existing body of literature as it pertains to CNS depressant withdrawal.

5.1: Toluene withdrawal decreases seizure threshold in mice

Initial pilot testing with the handling-induced seizure activity assay suggested that a higher dose, lower duration exposure model needed to be utilized. This finding is not surprising, and has been previously suggested by our group in reference to other models of toluene abuse in rodents (S. P. Callan et al., 2016). The most efficacious model appears to have been a relatively high

concentration of toluene (5,000 ppm) given for 24 hours. Attempts at utilizing an abuse-level concentration of toluene (5,000 ppm) for the 96 hours utilized by Evans and Balster in their study of 1,1,1-TCE withdrawal (E. B. Evans & Balster, 1993) did decrease seizure threshold, but resulted in symptoms of toxicity including soft tissue irritation and death. Reducing the exposure concentration (1,000 ppm) eliminated the deleterious effects but also did not significantly impact handling-induced seizure thresholds. Based on these results, the final concentration/duration combination was selected as a middle-ground between the models tested. The majority of the animals responded to the toluene exposure with clonic seizure activity within two hours of the cessation of exposure. In addition, the majority of the animals had stopped having seizures in response to the tail-twist by six hours post-exposure. It is worth noting that those mice that were still responding at six hours post-exposure were also the animals that took longest to begin initial responding to the handling protocol. No animals died or displayed signs of soft tissue irritation (e.g., red, swollen or scabbed eyelids and nostrils) following exposure. Interestingly, unlike the work by Evans and Balster (1993), the peak seizure frequency was not maintained across time, most likely due to the acute nature of the withdrawal paradigm (e.g., our present data displayed a pronounced peak in responding followed by a smooth decline, whereas Evans and Balster displayed prolonged responding).

The present results demonstrate that mice exposed and then removed from a protracted, acute abuse-level concentration of toluene vapor (5000 ppm) display behaviorally relevant seizure symptoms for several hours following removal from the exposure chamber. This represents the first such quantification of the timeline of hyper-excitability during toluene abstinence. The manifestation of handling-induced seizures is a commonly measured symptom of withdrawal from CNS depressant substances (Metten & Crabbe, 2005). As has been observed with ETOH

withdrawal (Prediger, da Silva, Batista, Bittencourt, & Takahashi, 2006), the mice tested in this experiment displayed heightened sensitivity to handling for a limited time following exposure, after which time the animals behaved like control animals. This limited window of susceptibility to excitation is a classic feature of drug withdrawal, and provides further support for the argument that the observed seizure behaviors were indeed the result of the abstinence itself.

The use of PTZ to induce seizure activity served to provide a higher degree of experimental control than handling-induced seizures alone. Additionally, PTZ provided a wider spectrum of seizure symptoms than handling, allowing for a more robust quantification and analysis of the effects of toluene abstinence. The use of PTZ (and handling-induced seizures) are well-characterized tools for studying changes in seizure threshold in rodent disease models (Crabbe, 1992; Goldstein, 1972a, 1972b). Of particular note is the reduction in seizure severity following 30-minute re-exposure to toluene. While it is important to note that toluene itself is anti-convulsant (Chan, Lee, & Chen, 2006; Wood, Coleman, Schuler, & Cox, 1984), a reduction in the severity of symptoms following re-exposure to drug is a classic indication that the symptoms were indeed withdrawal related and not the product of acute toxicity.

As previously mentioned, prior preclinical research examining withdrawal from inhaled solvents is scant. However, the current findings compare favorably to the limited work available. Both the present work and the prior work by Evans and Balster (1993) successfully induced physical withdrawal symptoms in mice following prolonged exposure to solvent vapor (toluene and TCE, respectively). In both cases, mice displayed handling-induced convulsions as well as increased responding to injections with PTZ. Both works were also able to ameliorate the effects of withdrawal by re-exposing animals to drug. As the authors of the prior work note, amelioration of a withdrawal symptom by re-exposure to drug is a classic trait of dependence-forming CNS

depressants. Additionally, our present results are consistent with what has been shown in the study of ETOH withdrawal. John Crabbe's extensive body of work has repeatedly demonstrated that mice display decreased seizure threshold during alcohol withdrawal (Crabbe, Belknap, & Buck, 1994; Greenberg & Crabbe, 2016).

However, our present work differs from prior reports in several ways. First, both TCE and ETOH withdrawal models commonly use especially protracted exposure durations (e.g., 96 hours). In our present work, we found that giving behaviorally relevant concentrations of toluene (i.e., 5,000 ppm) for much longer than 24 hours was deleterious to the health of the animals, while utilizing lower concentrations (e.g., 1,000 ppm) did not affect seizure thresholds (see pilot data, above). This difference from prior reports may be due to differences in the potency of these compounds. Evans and Balster (1993) specifically mention selecting TCE as their test compound as it less pharmacodynamically potent than toluene. Considering the decreased duration of exposure we used in this experiment, it is surprising that we demonstrated a higher percentage of handling-induced seizure responders than Evans and Balster (1993). Again, this could be a function of the differences between toluene and TCE. Additionally, Evans and Balster reported that 42 mg/kg of PTZ was a subthreshold dose for inducing seizures. We found that a small minority of control animals (*N = 1)* responded to this concentration (significantly less than responded to the 48mg/kg concentration). As both experiments used the same strain of mice, this difference is likely spurious.

It is also important to note that while ETOH withdrawal models also make use of longer exposure durations, due to alcohol's functionally zero-order metabolic traits, these models require extra adjunctive treatments with i.p. ETOH and alcohol dehydrogenase inhibitors (Crabbe, 1992). Additionally, many published reports of ETOH withdrawal utilize specially bred "withdrawal-

seizure prone" mice (Crabbe et al., 1994) to increase baseline responding, whereas we used unmodified animals. Despite this, the similarities between our results and the work of authors like John Crabbe (1994) is apparent.

A potential confounding factor is that of the decreased food and water intake observed by the animals (as typified by weight loss during exposure) alongside toluene's hypothermic properties (Paez-Martinez, Aldrete-Audiffred, et al., 2013). While the animals displayed statistically significant signs of weight catch-up across a 72-hour recovery window, indicating that the initial reduction in weight was not the result of permanent metabolic toxicity, the fact remains that the mice exposed to toluene vapor lost more weight than their control counterparts. At first examination, this difference (and its likely proximal cause—reduced food and water intake) may well represent confounds that could account for the differences in seizure susceptibility observed in this report. However, this does not appear to be the case. It is worth noting that prior evidence indicates that dehydration and food deprivation do not increase seizure thresholds; indeed, these states may be protective against seizures (Rowley et al., 2011; Andrew et al.,1989). Specifically, food deprivation appears to protect *against* seizures (LeBoeuf, Gruninger, & Garcia, 2007), and indeed food intake following fasting is a method of inducing seizures (Enginar, Nurten, Karamursel, Zengin, & Baran, 2010), as opposed to the fasting itself.

Likewise, dehydration appears to protect against seizure activity (Fetterman & Kumin, 1933; Kahn, Etienne, & Blum, 1979), though it is important to note that both hypernatremia and hyponatremia (imbalances of sodium concentration within the body) may precipitate seizures (Pasantes-Morales & Tuz, 2006), though significant changes in sodium concentrations are more commonly associated with exercise-induced dehydration (e.g., sweating) or over hydration (esp. in cases of psychiatric polydipsia). The mechanistic relationship between seizure activity and

hydration is a complex one, however it appears that mice absent Aquaporin-4 Water Channels, which play a role in the movement of water through glial cells, show increased seizure threshold (e.g., are less likely to seize) compared to wild type control animals (Binder et al., 2006), suggesting that decreased cellular hydration may be protective against the onset of seizures. Finally, hypothermia appears to be protective against seizures, and is even considered an adjunct treatment for refractory status epilepticus (Harbert et al., 2011; Wang, Liu, Li, Zhang, & Li, 2011). Considering that seizure activity is the result of unrestrained cellular excitation, perhaps it should not be surprising that these factors, all of which are typically associated with lowered basal activity both behaviorally and neurologically, would not engender convulsions.

Increased CNS excitation during withdrawal is a hallmark symptom of CNS depressants, and it comes as no surprise that we have demonstrated withdrawal-induced seizure activity in toluene-abstinent mice. The present work paints a clear picture that these seizures are indeed due to toluene abstinence. The possibility of over intoxication is ruled out be the significant amelioration of seizures during re-exposure. The time-course data for the handling-induced seizures rules out a permanent adverse change due to toxic tissue insult. The profile of the seizure response, including the transient window of susceptibility and a robust increase in the severity of seizures, is reminiscent of withdrawal from other drugs of abuse.

When considering the mechanisms behind withdrawal seizures, the excitatory glutamate system is the most likely candidate. PTZ is categorized as a “nonspecific pro-convulsant” without binding action at any receptor terminals, meaning that responding to PTZ does not indicate a likely receptor *per se*. However, as has been previously mentioned, toluene is a potent NMDAR antagonist (Riegel & French, 2002), much like ETOH. Withdrawal from ETOH precipitates seizures by increasing activity at Glutamate receptors during the abstinence period (Doremus,

Brunell, Varlinskaya, & Spear, 2003). Indeed, prior work has suggested that toluene exposure is protective against seizures produced by targeting NMDARs (Chan et al., 2006; Cruz et al., 2003). This result is consistent with our findings that re-exposure to toluene vapor ameliorated the effects of toluene abstinence on seizure severity, further implicating the glutamate system as the likely source of toluene withdrawal-related seizure activity. A seemingly plausible alternative hypothesis for the seizure symptoms observed herein would be serotonin syndrome. There has been some evidence that high doses of toluene can produce symptoms consistent with serotonin storm in rodents (Rivera-Garcia, Lopez-Rubalcava, & Cruz, 2015). A commonly used measure of serotonin syndrome-like behavior in mice is the "Head twitch response," which may at first consideration be confused with clonic seizure activity. However, it is important to note that the rhythmic head twitching produced by elevated levels of serotonin is categorically unlike clonus and that the clonic symptoms observed in our animals included both clonic and tonic symptoms, ruling out serotonin storm as the likely cause of these effects.

5.2: Toluene withdrawal leads to an anxiogenic response in mice: EPM

The main measure of anxiety-like behavior featured in this report was the Elevated Plus Maze. As predicted, acute toluene exposure increased open arm exploration without increasing the number of zone transitions or the total distance traveled. These results are similar to what we found with ETOH, whereby 2 g/kg increased open arm exploration without impacting overall activity. Twenty-four hours following a 24-hour exposure to 5,000 ppm toluene, abstinent mice spent a significantly lower proportion of time on the open arms compared to air controls. As with acute exposure, these animals did not differ from controls in terms of distance traveled or number of zone transitions. The 72 hour abstinence cohort, however, showed no significant differences from controls in terms of open arm preference, distance traveled, or zone transitions. Finally, re-

exposure to toluene vapor reversed the effects of toluene abstinence, producing an anxiolytic-like profile similar to what is seen with acutely applied toluene alone. It is worth noting, however, that the 24 hour air control group exposed to 30 minutes of 5,000 ppm toluene did show significantly reduced distances traveled compared to controls.

These results as a whole are highly suggestive of toluene's anxiolytic/anxiogenic properties. First, we were able to replicate work by Bowen and colleagues (1996) demonstrating that acutely administered toluene vapor is anxiolytic. Interestingly, in their report Bowen and colleagues found significantly elevated activity measures compared to controls, while in the present work we found that only open arm preference was increased. It is possible that this difference is due to detection method—the present work utilized automated tracking software, while Bowen and colleagues utilized manual tracking methods. Additionally, the maze used in the present experiment was plastic, while the maze used by Bowen and colleagues (1996) was lacquered wood. Indeed, the current results represent a stronger confirmation of Bowen's initial work, as increased activity during EPM exploration could indicate a locomotor confound. Despite these discrepancies, the main results stand—both papers have successfully demonstrated that toluene is anxiolytic as measured by the EPM. This result also compares favorably to work performed using the probe burying task (Páez-Martíinez, Cruz, & López-Rubalcava, 2003).

While there are no other extant reports demonstrating anxiety-like behavior during toluene abstinence, the results observed in mice 24 hours abstinent are consistent with human clinical reports (Perron et al., 2011). These results are highly congruent with work examining ETOH withdrawal, where the EPM has served as a gold standard assay for withdrawal-induced anxiety-like behavior for years (Doremus et al., 2003; Gonzaga et al., 2016; Lal, Prather, & Rezazadeh, 1993). The fact that toluene abstinence decreased open arm exploration without impacting overall

activity or exploratory-specific behavior (as typified by the number of zone transitions) strongly suggests a pure anxiety-like response to the abstinence itself. Indeed, the only point in which toluene exposure impacted locomotor behavior in the EPM was the 24 hour air control animals exposed acutely to 30 minutes toluene vapor. This result is strange, as these animals displayed the same anxiolytic response to toluene vapor demonstrated by the two other acutely exposed groups, yet they displayed significantly less activity while doing so. This may suggest that the repeated handling and environmental disruptions involved in the combination of the 24 hour exposure and 30 minute re-exposure may be a stressor to the animals, the effects of which are masked in the other 24-hour group by toluene withdrawal.

The recovery of the mice in the 72 hour condition, as indicated by the amelioration of the anxiety-like behaviors measured at 24 hours abstinence, suggests that the anxiety observed at 24-hours was not the result of damage due to toxicity or other permanent changes to physiology. Similar transient effects have been observed in the study of ETOH withdrawal (Prediger et al., 2006). This is consistent with the results of the toluene re-exposure paradigm, a test that other papers have used in the past to demonstrate that the symptoms observed are indeed related to the abstinence period itself (e.g., E. B. Evans & Balster, 1993). As was observed in the seizure studies, re-exposing animals to toluene reduced anxiety-like behavior, which strongly suggests that the abstinence itself was the likely cause of the increased open arm exploration. These two pieces of evidence provide strong convergent evidence that abstinence following prolonged toluene exposure produces behaviorally meaningful symptoms similar to what has been seen with ETOH and other, better characterized, drugs of abuse.

5.3: Toluene withdrawal leads to an anxiogenic response in mice: MBT, LDB, and OFT

The Marble Burying task was highly consistent with the EPM. Animals exposed to 2 g/kg

ETOH displayed significantly reduced burying behavior after 30 minutes compared to controls. These results were echoed with the acute toluene exposure group, who also buried significantly fewer marbles than control animals. Animals 24 hours abstinent from toluene, conversely, buried significantly more marbles during the 30 minute test period than controls, while animals 72 hours abstinent from toluene exposure did not differ significantly from controls in terms of number of marbles buried.

These results strongly support the EPM results above and represent the first known use the MBT to assess the anxiety profile of toluene exposure and abstinence in mice. Our positive control results with ETOH are consistent with prior literature (Gaikwad & Parle, 2011), confirming the validity of our model. It is important to note that two prior reports have used burying behavior in the context of toluene exposure, but both of these experiments utilized the defensive conditioned burying task, in which the object buried is an electrified probe (Lopez-Rubalcava, Hen, & Cruz, 2000; Páez-Martíinez et al., 2003). While similar in terms of the operant behavior measured (burying behavior), these two tasks are mechanistically disparate. The conditioned burying task is a learned response to a threat, while the MBT falls under the umbrella category "tests of unconditioned anxiety" (Ennaceur, 2014; Ennaceur & Chazot, 2016). Both methods have strengths and weaknesses, however for this report unconditioned tests were utilized in order to avoid the risk of confound due to toluene's effects on learning and memory (Win-Shwe & Fujimaki, 2012). Though these results represent the first time the MBT has been used with reference to toluene, our findings that toluene abstinent mice bury significantly more marbles than controls are consistent with prior reports examining ETOH withdrawal, in which abstinent animals show increased burying behavior compared to controls (Rose et al., 2016; Umathe, Bhutada, Dixit, & Shende,

2008). Indeed, this test appears to show good internal consistency with other unconditioned anxiety measures with regards to ETOH withdrawal (Perez & De Biasi, 2015).

Despite this, there is some debate as to *what*, exactly, the MBT is measuring. The test itself was first developed to take advantage of the natural burying behavior displayed by rodents in response to stressors such as novelty, offered as a more sensitive version of the then-standard grooming observation (Broekkamp, Rijk, Joly-Gelouin, & Lloyd, 1986). However, it quickly became a commonly used measure for screening anxiolytics (Nicolas, Kolb, & Prinssen, 2006) and antidepressants (Manna & Umathe, 2015). However, other researchers have argued that the MBT is a measure of perseverative behavior, a related-yet-distinct concept to anxiety (Joel, 2006; Prajapati, Kalaria, Karkare, Parmar, & Sheth, 2011).

Work by Thomas and colleagues (2009) examined the MBT alongside the OFT and LDB tests using multiple strains of mice. They found that strain differences in unaltered anxiety-like behavior correlated well between the OFT and LDB, but the MBT did not appear to be related to either of these tests. Additionally, the authors found that MBT burying behavior appeared to be unrelated to novelty, instead concluding that this behavior is reflective of perseverative behavior, not anxiety *per se*. However, Thomas and colleagues failed to consider that the OFT and LDB are both novel exploration-based assays, while the MBT is based off a completely different behavior (digging). Indeed, the original work utilizing the MBT considered it an analogue to grooming behavior (itself a measure of both anxiety and perseveration), not locomotor behavior (Broekkamp et al., 1986). Thus, it is not surprising that the OFT behavior that correlated highest with the MBT was stereotypy ($r = 0.62$). While the authors noted that this was a "trending" result ($p = 0.056$), it is important to consider that their sample size (the standard 10-12 per group) may have been insufficient for a correlation analysis. More importantly, the MBT used in this dissertation was

consistent with three other tests of anxiety for the detection of both anxiogenic and anxiolytic behavior in mice using two separate drugs and three separate time points. As such, it is appropriate to consider the MBT a test of novelty-independent anxiety *and* perseverative behavior, and no attempt was made to distinguish between the two.

Similar to the MBT, the Light Dark Box (LDB) provided results congruent with the EPM. Animals acutely exposed to ETOH displayed increased Light side exploration without a change in latency to first entering the dark chamber. Acute toluene-exposed mice however, showed a different profile. Mice exposed to 30 min of toluene did not differ from controls in terms of how much time they spent in the lit chamber. They did, however, display a significantly higher latency to first entering the darkened area and a significantly reduced number of zone transitions as well as significantly reduced distances traveled. Animals 24-hours abstinent from toluene, however, displayed decreased lit-box preference and decreased latency to enter the dark zone. These effects were not present when the animals were tested at 72 hours, though it is worth noting that toluene-exposed animals in the 72 hour condition did display significantly reduced zone transitions compared to air controls. Despite this, the 72-hour abstinent mice did not differ from controls in either time spent in the lit compartment or the latency to first entering the dark box, the two measures associated with anxiety-like behavior.

Of the four anxiety measures utilized in this experiment, the Light Dark Box was the least congruent with the others. While the core hypotheses were correct (toluene abstinence produced transient anxiogenic behavior at 24 but not 72 hours abstinence), our ETOH and toluene positive controls displayed converging (but not identical) signs of anxiolysis. Ethanol exposure produced the expected increase in lit box preference, while acute toluene exposure increased the latency to first entering the darkened box. This may indicate different forms of anxiolysis. It is also possible

that 5,000 ppm toluene produces a more mild form of anxiety relief than 2 g/kg ETOH, a level below the threshold for detection in the LDB. This is a concept we see mirrored in the OFT (discussed below).

Our LDB apparatus produced a more mild form of light aversion than has been reported by some other recent studies, despite similar lighting features (Garcia-Gutierrez et al., 2016; Laureano-Melo et al., 2016). However, this is likely due to differences in protocol; starting a mouse on the lit (as was done in the present work) or darkened side (as was done in the above studies) of the apparatus appears to affect lit side preference (Bourin & Hascoet, 2003) such that placing animals on the lit side results in increased lit side preference (Kulesskaya & Voikar, 2014). Despite this, we chose to start our animals on the lit side of the box to be consistent with the original design of the LDB protocol (Bourin & Hascoet, 2003 for a review). The LDB is a highly variable test of anxiety, and relatively small differences in testing environment (e.g., color of the floor, orientation of the animal in the testing chamber, ratio size of lit to dark chambers, strain of mouse, and light/dark cycle) can affect animal performance on the task (Bourin, 2015; Kulesskaya & Voikar, 2014). Ultimately, it is important to note that our model was able to successfully elicit and measure both anxiolysis and anxiogenesis in mice, suggesting that our LDB protocol was both valid and sufficient to the task.

The results of the Open Field Test (OFT) partially support the EPM. While acutely exposed mice did not differ in terms of center square exploration, mice abstinent for 24 hours did show significant reductions in time spent in the center square, a reduction not present in mice tested at 72 hours post-exposure. It is important to note that toluene-exposed mice tested in the OFT did not differ from air controls in total distance traveled at any of the time points tested. This suggests that the anxiogenic behavior observed in the 24-hour abstinent animals was not confounded by changes

in basal locomotor rate. This evidence is congruent with what we observed with the EPM and LDB, and provides further evidence that what we have observed herein is indeed anxiety-like behavior.

When taken as a whole, the four anxiety tests used in this study provide largely convergent evidence that toluene is acutely anxiolytic while being transiently anxiogenic during withdrawal. The MBT, LDB, and OFT all support the initial findings with EPM. Indeed, these findings are consistent with reports that acute toluene exposure has anxiolytic properties (Páez-Martíinez et al., 2003; Paez-Martinez, Flores-Serrano, Ortiz-Lopez, & Ramirez-Rodriguez, 2013), though these results remain the first evidence that withdrawal from a solvent (including toluene) produces anxiogenic behavior in rodents. These overall results are consistent with human case reports of inhalant withdrawal precipitating anxiety (Basu, Jhirwal, Singh, Kumar, & Mattoo, 2004; Kouzoupis et al., 2010; Perron et al., 2011). Moreover, these results represent the first time the Light Dark Box and the Marble Burying Test have been used in the study of toluene abuse.

When qualifying the anxiety effects observed herein, it is important to note that while food and water deprivation are sometimes used as a model of chronic mild stress, 24 hour deprivation followed by free access to food and water does not appear to be sufficient to produce an anxious phenotype in rats, though it does increase motivated behaviors (Dietze, Lees, Fink, Brosda, & Voigt, 2016). Indeed, there is some evidence that chronic (multi-month) mild dehydration may even increase light-side exploration in the LDB (Elgot, El hiba, & Gamrani, 2012), though these authors did not report measures of activity, making it impossible to separate anxiety-like behavior from activity due to water seeking.

Anxiety-like behaviors in mice are commonly confounded by changes in basal locomotor rates. However, the anxiogenic effects observed in this study appear to be independent of an

underlying locomotor effect, though acute toluene exposure does appear to reduce activity in the LDB. As this was the only test in which reduced activity was observed in response to acute toluene exposure alone, it is unlikely that the anxiolysis observed in response to acute toluene exposure was the result of locomotor impairments.

Unlike with the seizure responses observed in this report, the presence of anxiety-related phenotypes in response to toluene exposure and abstinence is relatively straightforward. As has been previously stated, the anxiolytic-like properties of toluene intoxication are similar to what has been observed with other compounds active at GABA receptors (Bowen, Wiley, Jones, & Balster, 1999). Anxiety as a withdrawal symptom is a GABA-mediated process that is known to increase the risk of drug-related relapse (Fox, Bergquist, Hong, & Sinha, 2007) and to moderate increased alcohol consumption during withdrawal states (Koob, 2009). Specifically, increased GABA release in the amygdala appears to mediate anxiety during alcohol withdrawal (Roberto et al., 2010). This process is also typically governed by increased NMDAR activity during chronic alcohol withdrawal, though this does not appear to always be the case for alcohol withdrawal (Van Skike, Diaz-Granados, & Matthews, 2015). Furthermore, alcohol withdrawal-mediated anxiety behavior appears to be related to increases in corticotrophin-releasing factor (Valdez et al., 2002), most likely through increases in GABA release in the amygdala (per Roberto et al., 2010).

5.4: Overall conclusions and future directions

Our present results provide strong convergent validity to the concept that we are actually measuring the effects of toluene withdrawal. That is to say, our model permits us to exclude several likely alternative hypotheses and provides confidence that what we have measured in these experiments is indeed the result of withdrawal. First, we ruled out the likelihood of a toxic "overdose" reaction by demonstrating that re-exposure to toluene reduces both anxiety-like

behavior and seizure severity instead of increasing both as would be expected of a drug overdose. Second, we ruled out the likelihood of a long lasting toxic impairment (e.g., damage to, or destruction of, neurons) by demonstrating that the effects on both anxiety and seizure are transient. Additionally, the significant catch-up weight gain by the toluene exposed mice suggests that no lasting physiological harm was done to the animals. Third, we observed no confounds to our anxiety measures due to locomotor behavior, which further increases our confidence. Despite this, there were limitations to the present design. While the mice showed signs of weight catchup, we cannot totally rule out a mediating role of hypo- /hypernatremia in the seizures displayed by abstinent mice. Second, the present exposure model, while highly efficient, was not a face valid simulation of the human abuse experience. In order to gain the mechanistic control required to test for withdrawal syndromes, we resorted to a highly artificial method of exposure. This limits the direct applicability of our findings to the human medical field.

In conclusion, this was the preclinical evidence for a toluene-specific withdrawal syndrome in a mouse model of toluene abuse. These results open up a number of exciting future possibilities and hypotheses. First, it is important to note that the present work represented a single facet of withdrawal—acute withdrawal. A logical next step in elucidating the consequences of toluene abstinence would be to utilize a longer chronic exposure model to better imitate the kindling effect seen in real inhalant abusers. Likewise, the face validity of this model would be improved by including female animals as well as adolescents. Second, the present findings should be expanded by examining other purported symptoms of toluene withdrawal, especially aggression and depression. Third, the present work suggest several molecular targets by which toluene may be producing the effects discussed herein. Future studies should seek to elucidate the mechanism(s) by which toluene abstinence produces anxiogenic behavior and lowers seizure threshold.

Understanding these mechanisms is a critical jumping off point for developing targeted therapies to assist individuals dependent on inhalants.

REFERENCES

Akoijam, B., Jamir, M., Phesao, E., & Senjam, G. (2013). Inhalant use among schoolchildren in Northeast India: a preliminary study. . *Substance abuse: research and treatment, 7*, 185.

ATSDR. (2000). *Toxicological profile for toluene*. Atlanta: U.S. Public Health Service.

Bale, A. S., Tu, Y., Carpenter-Hyland, E. P., Chandler, L. J., & Woodward, J. J. (2005). Alterations in glutamatergic and gabaergic ion channel activity in hippocampal neurons following exposure to the abused inhalant toluene. *Neuroscience, 130*(1), 197-206.

Ballenger, J. C., & Post, R. M. (1978). Kindling as a model for alcohol withdrawal syndromes. *Br J Psychiatry, 133*, 1-14.

Basu, D., Jhirwal, O. P., Singh, J., Kumar, S., & Mattoo, S. K. (2004). Inhalant abuse by adolescents: a new challenge for Indian physicians. *Indian J Med Sci, 58*(6), 245-249.

Batis, J. C., Hannigan, J. H., & Bowen, S. E. (2010). Differential effects of inhaled toluene on locomotor activity in adolescent and adult rats. *Pharmacol Biochem Behav, 96*, 438-448. doi:S0091-3057(10)00204-2 [pii]

10.1016/j.pbb.2010.07.003

Beasley, M., Frampton, L., & Fountain, J. (2006). Inhalant abuse in New Zealand. *N Z Med J, 119*(1233), U1952.

Becker, H. C. (1998). Kindling in alcohol withdrawal. *Alcohol Health Res World, 22*(1), 25-33.

Becker, H. C., Diaz-Granados, J. L., & Weathersby, R. T. (1997). Repeated ethanol withdrawal experience increases the severity and duration of subsequent withdrawal seizures in mice. *Alcohol, 14*(4), 319-326.

Becker, H. C., & Hale, R. L. (1993). Repeated episodes of ethanol withdrawal potentiate the severity of subsequent withdrawal seizures: an animal model of alcohol withdrawal "kindling". *Alcohol Clin Exp Res, 17*(1), 94-98.

Becker, H. C., Veatch, L. M., & Diaz-Granados, J. L. (1998). Repeated ethanol withdrawal experience selectively alters sensitivity to different chemoconvulsant drugs in mice. *Psychopharmacology (Berl), 139*(1-2), 145-153.

Beckstead, M. J., Weiner, J. L., Eger, E. I., 2nd, Gong, D. H., & Mihic, S. J. (2000). Glycine and gamma-aminobutyric acid(A) receptor function is enhanced by inhaled drugs of abuse. *Mol Pharmacol, 57*(6), 1199-1205.

Binder, D., Yao, X., Zador, Z., Sick, T., Verkman, A., & Manley, G. (2006). Increased Seizure Duration and Slowed Potassium Kinetics in Mice Lacking Aquaporin-4 Water Channels. *Glia, 56*, 631-636.

Bourin, M. (2015). Animal models for screening anxiolytic-like drugs: a perspective. *Dialogues Clin Neurosci, 17*(3), 295-303.

Bourin, M., & Hascoet, M. (2003). The mouse light/dark box test. *Eur J Pharmacol, 463*(1-3), 55-65.

Bowen, S. E. (2011). Two serious and challenging medical complications associated with volatile substance misuse: sudden sniffing death and fetal solvent syndrome. *Subst Use Misuse, 46 Suppl 1*, 68-72. doi:10.3109/10826084.2011.580220

Bowen, S. E., & Balster, R. L. (1996). Effects of inhaled 1,1,1-trichloroethane on locomotor activity in mice. *Neurotoxicol Teratol, 18*(1), 77-81. doi:10.1016/0892-0362(95)02024-1

Bowen, S. E., & Balster, R. L. (1998). A direct comparison of inhalant effects on locomotor activity and schedule-controlled behavior in mice. *Exp Clin Psychopharmacol, 6*(3), 235-247. doi:10.1037/1064-1297.6.3.235

Bowen, S. E., Batis, J. C., Paez-Martinez, N., & Cruz, S. L. (2006). The last decade of solvent research in animal models of abuse: mechanistic and behavioral studies. *Neurotoxicol Teratol, 28*(6), 636-647. doi:10.1016/j.ntt.2006.09.005

Bowen, S. E., Charlesworth, J. D., Tokarz, M. E., Wright, M. J., Jr., & Wiley, J. L. (2007). Decreased sensitivity in adolescent vs. adult rats to the locomotor activating effects of toluene. *Neurotoxicol Teratol, 29*(6), 599-606. doi:S0892-0362(07)00298-X [pii]
10.1016/j.ntt.2007.08.001

Bowen, S. E., & Cruz, S. L. (2014). Inhalants: addiction and toxic effects in the human. In B. Madras & M. Kuhar (Eds.), *The effects of drug abuse on the human nervous system* (Vol. 2, pp. 553-569.). Oxford, UK: Academic Press.

Bowen, S. E., Daniel, J., & Balster, R. L. (1999). Deaths associated with inhalant abuse in Virginia from 1987 to 1996. *Drug Alcohol Depend, 53*(3), 239-245. doi:10.1016/S0376-8716(98)00139-2

Bowen, S. E., Kimar, S., & Irtenkauf, S. (2010). Comparison of toluene-induced locomotor activity in four mouse strains. *Pharmacol Biochem Behav, 95*(2), 249-257. doi:10.1016/j.pbb.2010.01.014

Bowen, S. E., Wiley, J. L., & Balster, R. L. (1996). The effects of abused inhalants on mouse behavior in an elevated plus-maze. *Eur J Pharmacol, 312*(2), 131-136. doi:10.1016/0014-2999(96)00459-1

Bowen, S. E., Wiley, J. L., Evans, E. B., Tokarz, M. E., & Balster, R. L. (1996). Functional observational battery comparing effects of ethanol, 1,1,1-trichloroethane, ether, and flurothyl. *Neurotoxicol Teratol, 18*(5), 577-585. doi:10.1016/0892-0362(96)00064-5

Bowen, S. E., Wiley, J. L., Jones, H. E., & Balster, R. L. (1999). Phencyclidine- and diazepam-like discriminative stimulus effects of inhalants in mice. *Exp Clin Psychopharmacol, 7*(1), 28-37. doi:10.1037/1064-1297.7.1.28

Bray, H. G., Thorpe, W. V., & White, K. (1950). Kinetic studies of the metabolism of foreign organic compounds. *Biochem J, 47*(2), xiii.

Broekkamp, C., Rijk, H., Joly-Gelouin, D., & Lloyd, K. (1986). Major tranquillizers can be distinguished from minor tranquillizers on the basis of effects on marble burying and swim-induced grooming in mice. *Eur J Pharmacol, 126*(3), 223-229.

Bruckner, J. V., & Peterson, R. G. (1981). Evaluation of toluene and acetone inhalant abuse. II. Model development and toxicology. *Toxicol Appl Pharmacol, 61*(3), 302-312.

Butland, B. K., Field-Smith, M. E., Ramsey, J. D., & Anderson, H. R. (2012). Twenty-five years of volatile substance abuse mortality: a national mortality surveillance programme. *Addiction*. doi:10.1111/j.1360-0443.2012.04047.x

Callan, S. P., Kott, J. M., Cleary, J. P., McCarthy, M. K., Baltes, B. B., & Bowen, S. E. (2016). Changes in developmental body weight as a function of toluene exposure: A meta-analysis of animal studies. *Hum Exp Toxicol, 35*(4), 341-352. doi:10.1177/0960327115591377

Callan, S. P., Kott, J.M., Cleary, J.P., McCarthy, M.K., Baltes, B.B., Bowen, S.E. (2015). Changes in Developmental Body Weight as a Function of Toluene Exposure: A Meta-Analysis of Animal Studies. *Hum Exp Toxicol*.

Chan, M. H., Chung, S. S., Stoker, A. K., Markou, A., & Chen, H. H. (2012). Sarcosine attenuates toluene-induced motor incoordination, memory impairment, and hypothermia but not brain stimulation reward enhancement in mice. *Toxicol Appl Pharmacol, 265*(2), 158-165. doi:10.1016/j.taap.2012.10.004

Chan, M. H., Lee, C. C., & Chen, H. H. (2006). Effects of toluene on seizures induced by convulsants acting at distinct ligand-gated ion channels. *Toxicol Lett, 160*(3), 179-184. doi:10.1016/j.toxlet.2005.07.002

Chen, F., Jarrott, B., & Lawrence, A. J. (1999). Up-regulation of cortical AMPA receptor binding in the fawn-hooded rat following ethanol withdrawal. *Eur J Pharmacol, 384*(2-3), 139-146.

Cooper, E., & Vernon, J. (2013). The effectiveness of pharmacological approaches in the treatment of alcohol withdrawal syndrome (AWS): a literature review. *J Psychiatr Ment Health Nurs, 20*(7), 601-612. doi:10.1111/j.1365-2850.2012.01958.x

Crabbe, J. C. (1992). Antagonism of ethanol withdrawal convulsions in withdrawal seizure prone mice by diazepam and abecarnil. *Eur J Pharmacol, 221*, 85-90.

Crabbe, J. C., Belknap, J. K., & Buck, K. J. (1994). Genetic animal models of alcohol and drug abuse. *Science, 264*(5166), 1715-1723.

Crabbe, J. C., Kendler, K. S., & Hitzemann, R. J. (2013). Modeling the diagnostic criteria for alcohol dependence with genetic animal models. *Curr Top Behav Neurosci, 13*, 187-221. doi:10.1007/7854_2011_162

Criswell, H. E., & Breese, G. R. (2005). A conceptualization of integrated actions of ethanol contributing to its GABAmimetic profile: a commentary. *Neuropsychopharmacology, 30*(8), 1407-1425. doi:10.1038/sj.npp.1300750

Cruz, S. L. (2011). The latest evidence in the neuroscience of solvent misuse: an article written for service providers. *Subst Use Misuse, 46 Suppl 1*, 62-67. doi:10.3109/10826084.2011.580215

Cruz, S. L., & Dominguez, M. (2011). Misusing volatile substances for their hallucinatory effects: a qualitative pilot study with Mexican teenagers and a pharmacological discussion of their hallucinations. *Subst Use Misuse, 46 Suppl 1*, 84-94. doi:10.3109/10826084.2011.580222

Cruz, S. L., Gauthereau, M. Y., Camacho-Munoz, C., Lopez-Rubalcava, C., & Balster, R. L. (2003). Effects of inhaled toluene and 1,1,1-trichloroethane on seizures and death produced by N-methyl-D-aspartic acid in mice. *Behav Brain Res, 140*(1-2), 195-202.

Darker, C. D., Sweeney, B. P., Barry, J. M., Farrell, M. F., & Donnelly-Swift, E. (2015). Psychosocial interventions for benzodiazepine harmful use, abuse or dependence. *Cochrane Database Syst Rev, 5*, CD009652. doi:10.1002/14651858.CD009652.pub2

Dieterich, S. E., Stanley, L. R., Swaim, R. C., & Beauvais, F. (2013). Outcome expectancies, descriptive norms, and alcohol use: American Indian and white adolescents. *J Prim Prev, 34*(4), 209-219. doi:10.1007/s10935-013-0311-6

Dietze, S., Lees, K. R., Fink, H., Brosda, J., & Voigt, J. P. (2016). Food Deprivation, Body Weight Loss and Anxiety-Related Behavior in Rats. *Animals (Basel), 6*(1). doi:10.3390/ani6010004

Donald, J. M., Hooper, K., & Hopenhayn-Rich, C. (1991). Reproductive and developmental toxicity of toluene: a review. *Environ Health Perspect, 94*, 237-244.

Doremus, T. L., Brunell, S. C., Varlinskaya, E. I., & Spear, L. P. (2003). Anxiogenic effects during withdrawal from acute ethanol in adolescent and adult rats. *Pharmacol Biochem Behav, 75*(2), 411-418.

Duncan, J. R., Gibbs, S. J., & Lawrence, A. J. (2014). Chronic intermittent toluene inhalation in adolescent rats alters behavioural responses to amphetamine and MK801. *Eur Neuropsychopharmacol, 24*(3), 480-486. doi:10.1016/j.euroneuro.2013.06.001

Elgot, A., El hiba, O., & Gamrani, H. (2012). The anxiogenic-like effects of dehydration in a semi-desert rodent Meriones shawi indicating the possible involvement of the serotoninergic system. *Acta Histochem, 114*(6), 603-607. doi:10.1016/j.acthis.2011.11.005

Enginar, N., Nurten, A., Karamursel, Y., Zengin, A., & Baran, E. (2010). Scopolamine-induced convulsions in fasted mice after food intake: evaluation of the sedative effect in the suppression of convulsions. *Epilepsy Research, 89*(1), 2-6.

Ennaceur, A. (2014). Tests of unconditioned anxiety - pitfalls and disappointments. *Physiol Behav, 135*, 55-71. doi:10.1016/j.physbeh.2014.05.032

Ennaceur, A., & Chazot, P. L. (2016). Preclinical animal anxiety research - flaws and prejudices. *Pharmacol Res Perspect, 4*(2), e00223. doi:10.1002/prp2.223

Evans, A. C., & Raistrick, D. (1987). Patterns of use and related harm with toluene-based adhesives and butane gas. *Br J Psychiatry, 150*, 773-776.

Evans, E. B., & Balster, R. L. (1993). Inhaled 1,1,1-trichloroethane-produced physical dependence in mice: effects of drugs and vapors on withdrawal. *J Pharmacol Exp Ther, 264*(2), 726-733.

Fetterman, J., & Kumin, H. (1933). Dehydration in Epilepsy. *JAMA, 100*(13), 1005-1007.

Field-Smith, M. E. (2002). Trends in death associated with abuse of volatile substances. *need more info here*.

Flanagan, R. J., & Ives, R. J. (1994). Volatile substance abuse. *Bull Narc, 46*(2), 49-78.

Fox, H. C., Bergquist, K. L., Hong, K. I., & Sinha, R. (2007). Stress□induced and alcohol cue□induced craving in recently abstinent alcohol□dependent individuals. . *Alcoholism, 31*(3), 395-403.

Gaikwad, U., & Parle, M. (2011). Combination of aripiprazole and ethanol attenuates marble-burying behavior in mice. *Acta Pol Pharm, 68*(3), 435-440.

Garcia-Gutierrez, M. S., Navarrete, F., Aracil, A., Bartoll, A., Martinez-Gras, I., Lanciego, J. L., . . . Manzanares, J. (2016). Increased vulnerability to ethanol consumption in adolescent maternal separated mice. *Addict Biol, 21*(4), 847-858. doi:10.1111/adb.12266

Garland, E. L., & Howard, M. O. (2012). Volatile substance misuse. *Neuropharmacology*.

Goldstein, D. B. (1972a). An animal model for testing effects of drugs on alcohol withdrawal reactions. *J Pharmacol Exp Ther, 183*(1), 14-22.

Goldstein, D. B. (1972b). Relationship of alcohol dose to intensity of withdrawal signs in mice. *J Pharmacol Exp Ther, 180*(2), 203-215.

Gonzaga, N. A., Batistela, M. R., Padovan, D., de Martinis, B. S., Tirapelli, C. R., & Padovan, C. M. (2016). Ethanol withdrawal induces anxiety-like effects: Role of nitric oxide synthase in the dorsal raphe nucleus of rats. *Alcohol, 52*, 1-8. doi:10.1016/j.alcohol.2016.02.001

Greenberg, G. D., & Crabbe, J. C. (2016). Gene Targeting Studies of Hyperexcitability and Affective States of Alcohol Withdrawal in Rodents. *International review of neurobiology, 126*, 357-390. doi:10.1016/bs.irn.2016.02.010

Harbert, M. J., Tam, E. W., Glass, H. C., Bonifacio, S. L., Haeusslein, L. A., Barkovich, A. J., . . . Ferriero, D. M. (2011). Hypothermia is correlated with seizure absence in perinatal stroke. *J Child Neurol, 26*(9), 1126-1130. doi:10.1177/0883073811408092

Hass, U., Lund, S. P., Hougaard, K. S., & Simonsen, L. (1999). Developmental neurotoxicity after toluene inhalation exposure in rats. *Neurotoxicol Teratol, 21*(4), 349-357.

Howard, M. O., Bowen, S. E., Garland, E. L., Perron, B. E., & Vaughn, M. G. (2011). Inhalant use and inhalant use disorders in the United States. *Addict Sci Clin Pract, 6*(1), 18-31.

Jasova, D., Bob, P., & Fedor-Freybergh, P. (2007). Alcohol craving, limbic irritability, and stress. *Med Sci Monit, 13*(12), CR543-547.

Joel, D. (2006). Current animal models of obsessive compulsive disorder: a critical review. *Prog Neuropsychopharmacol Biol Psychiatry, 30*(3), 374-388. doi:10.1016/j.pnpbp.2005.11.006

Johnston, L. D., O'Malley, P. M., Miech, R. A., Bachman, J. G., & Schulenberg, J. E. (2015). Monitoring the Future national survey results on drug use: 1975-2014: Overview, key findings on adolescent drug use. *Ann Arbor: Institute for Social Research, The University of Michigan.*

Kahn, A., Etienne, B., & Blum, D. (1979). Controlled fall in natremia and risk of seizures in hypertonic dehydration. *Intensive care medicine, 5*(1), 27-31.

Kann, L., Kinchen, S., Shanklin, S. L., Flint, K. H., Kawkins, J., Harris, W. A., . . . Prevention. (2014). Youth risk behavior surveillance--United States, 2013. *MMWR Surveill Summ, 63 Suppl 4*, 1-168.

Keriotis, A. A., & Upadhyaya, H. P. (2000). Inhalant dependence and withdrawal symptoms. *J Am Acad Child Adolesc Psychiatry, 39*(6), 679-680. doi:10.1097/00004583-200006000-00004

Koga, K. (1978). [Distribution, metabolism and excretion of toluene in mice (author's transl)]. *Nihon Yakurigaku Zasshi, 74*(6), 687-698.

Kono, J., Miyata, H., Ushijima, S., Yanagita, T., Miyasato, K., Ikawa, G., & Hukui, K. (2001). Nicotine, alcohol, methamphetamine, and inhalant dependence: a comparison of clinical features with the use of a new clinical evaluation form. *Alcohol, 24*(2), 99-106.

Koob, G. F. (2009). Brain stress systems in the amygdala and addiction. *Brain Res, 1293*, 61-75. doi:10.1016/j.brainres.2009.03.038

Kosobud, A., & Crabbe, J. C. (1986). Ethanol withdrawal in mice bred to be genetically prone or resistant to ethanol withdrawal seizures. *J Pharmacol Exp Ther, 238*(1), 170-177.

Kouzoupis, A. V., Konstantakopoulos, G., Oulis, P., Kalfakis, N., & Papageorgiou, S. G. (2010). A case of severe toluene withdrawal syndrome treated with clonazepam. *J Neuropsychiatry Clin Neurosci, 22*(1). doi:10.1176/appi.neuropsych.22.1.123-j.e16.

Kulesskaya, N., & Voikar, V. (2014). Assessment of mouse anxiety-like behavior in the light-dark box and open-field arena: role of equipment and procedure. *Physiol Behav, 133*, 30-38. doi:10.1016/j.physbeh.2014.05.006

Kumar, S., Grover, S., Kulhara, P., Mattoo, S. K., Basu, D., Biswas, P., & Shah, R. (2008). Inhalant abuse: A clinic-based study. *Indian J Psychiatry, 50*(2), 117-120. doi:10.4103/0019-5545.42399

Lal, H., Prather, P. L., & Rezazadeh, S. M. (1993). Potential role of 5HT1C and/or 5HT2 receptors in the mianserin-induced prevention of anxiogenic behaviors occurring during ethanol withdrawal. *Alcohol Clin Exp Res, 17*(2), 411-417.

Laureano-Melo, R., da Silveira, A. L., de Azevedo Cruz Seara, F., da Conceicao, R. R., da Silva-Almeida, C., Marinho, B. G., . . . Cortes, W. D. (2016). Behavioral profile assessment in offspring of Swiss mice treated during pregnancy and lactation with caffeine. *Metab Brain Dis*. doi:10.1007/s11011-016-9847-5

LeBoeuf, B., Gruninger, T., & Garcia, L. (2007). Food deprivation attenuates seizures through CaMKII and EAG K+ channels. *PLOS Genet, 3*(9), 156.

Lister, R. G. (1987). The use of a plus-maze to measure anxiety in the mouse. *Psychopharmacology (Berl), 92*(2), 180-185.

Lo, P. S., Wu, C. Y., Sue, H. Z., & Chen, H. H. (2009). Acute neurobehavioral effects of toluene: involvement of dopamine and NMDA receptors. *Toxicology, 265*(1-2), 34-40. doi:10.1016/j.tox.2009.09.005

Lopez-Rubalcava, C., Hen, R., & Cruz, S. L. (2000). Anxiolytic-like actions of toluene in the burying behavior and plus-maze tests: differences in sensitivity between 5-HT(1B) knockout and wild-type mice. *Behav Brain Res, 115*(1), 85-94. doi:S0166-4328(00)00241-2 [pii]

Lovinger, D. M. (1993). Excitotoxicity and alcohol-related brain damage. *Alcohol Clin Exp Res, 17*(1), 19-27.

Lubman, D. I., Yucel, M., & Lawrence, A. J. (2008). Inhalant abuse among adolescents: neurobiological considerations. *Br J Pharmacol, 154*(2), 316-326. doi:bjp200876 [pii] 10.1038/bjp.2008.76

MacIver, M. B. (2009). Abused inhalants enhance GABA-mediated synaptic inhibition. *Neuropsychopharmacol, 34*(10), 2296-2304. doi:npp200957 [pii]

10.1038/npp.2009.57

Manna, S. S., & Umathe, S. N. (2015). Paracetamol potentiates the antidepressant-like and anticompulsive-like effects of fluoxetine. *Behav Pharmacol, 26*(3), 268-281. doi:10.1097/FBP.0000000000000104

Maxwell, J. C. (2001). Deaths related to the inhalation of volatile substances in Texas: 1988-1998. *Am J Drug Alcohol Abuse, 27*(4), 689-697.

McDermott, M. J., Drescher, C. F., Smitherman, T. A., Tull, M. T., Heiden, L., Damon, J. D., . . . Young, J. (2013). Prevalence and sociodemographic correlates of lifetime substance use among a rural and diverse sample of adolescents. *Subst Abus, 34*(4), 371-380. doi:10.1080/08897077.2013.776000

Metten, P., & Crabbe, J. C. (2005). Alcohol withdrawal severity in inbred mouse (Mus musculus) strains. *Behav Neurosci, 119*(4), 911-925. doi:10.1037/0735-7044.119.4.911

Mills, W. J., Grigg, B. J., Offermann, F. J., Gustin, B. E., & Spingarn, N. E. (2012). Engineering case report. Toluene and methyl ethyl ketone exposure from a commercially available contact adhesive. *J Occup Environ Hyg, 9*(5), D95-102. doi:10.1080/15459624.2012.676981

Moser, V. C., & Balster, R. L. (1985). Acute motor and lethal effects of inhaled toluene, 1,1,1-trichloroethane, halothane, and ethanol in mice: effects of exposure duration. *Toxicol Appl Pharmacol, 77*(2), 285-291. doi:0041-008X(85)90328-X [pii]

Muralidharan, K., Rajkumar, R. P., Mulla, U., Nayak, R. B., & Benegal, V. (2008). Baclofen in the management of inhalant withdrawal: a case series. *Prim Care Companion J Clin Psychiatry, 10*(1), 48-51.

Narayanaswamy, J. C., Viswanath, B., Ravi, M., & Muralidharan, K. (2012). Inhalant dependence: data from a tertiary care center in South India. *Indian J Psychol Med, 34*(3), 232-236. doi:10.4103/0253-7176.106017

Nicolas, L. B., Kolb, Y., & Prinssen, E. P. (2006). A combined marble burying-locomotor activity test in mice: a practical screening test with sensitivity to different classes of anxiolytics and antidepressants. *Eur J Pharmacol, 547*(1-3), 106-115. doi:10.1016/j.ejphar.2006.07.015

Niederhofer, H. (2007). Treating inhalant abuse with buspirone. *Am J Addict, 16*(1), 69. doi:10.1080/10550490601080126

Páez-Martíinez, N., Cruz, S. L., & López-Rubalcava, C. (2003). Comparative study of the effects of toluene, benzene, 1,1,1-trichloroethane, diethyl ether, and flurothyl on anxiety and nociception in mice. *Toxicol Appl Pharmacol, 193*(1), 9-16. doi:10.1016/s0041-008x(03)00335-1

Paez-Martinez, N., Aldrete-Audiffred, J., Gallardo-Tenorio, A., Castro-Garcia, M., Estrada-Camarena, E., & Lopez-Rubalcava, C. (2013). Participation of GABAA, GABAB receptors and neurosteroids in toluene-induced hypothermia: Evidence of concentration-ependent differences in the mechanism of action. *Eur J Pharmacol, 698*, 178-185.

Paez-Martinez, N., Flores-Serrano, Z., Ortiz-Lopez, L., & Ramirez-Rodriguez, G. (2013). Environmental enrichment increases doublecortin-associated new neurons and decreases

neuronal death without modifying anxiety-like behavior in mice chronically exposed to toluene. *Behav Brain Res, 256*, 432-440. doi:10.1016/j.bbr.2013.09.007

Pasantes-Morales, H., & Tuz, K. (2006). Volume changes in neurons: hyperexcitability and neuronal death. *Contrib Nephrol, 152*, 221-240. doi:10.1159/000096326

Patel, G. (2014). The management of substance abuse in the critically ill. *Dis Mon, 60*(8), 429-441. doi:10.1016/j.disamonth.2014.05.003

Pellow, S., Chopin, P., File, S. E., & Briley, M. (1985). Validation of open:closed arm entries in an elevated plus-maze as a measure of anxiety in the rat. *J Neurosci Methods, 14*(3), 149-167.

Perez, E., & De Biasi, M. (2015). Assessment of affective and somatic signs of ethanol withdrawal in C57BL/6J mice using a short term ethanol treatment. *Alcohol, 49*(3), 237-243.

Perit, K. E., Gmaz, J. M., Caleb Browne, J. D., Matthews, B. A., Dunn, M. B., Yang, L., . . . McKay, B. E. (2012). Distribution of c-Fos immunoreactivity in the rat brain following abuse-like toluene vapor inhalation. *Neurotoxicol Teratol, 34*(1), 37-46. doi:10.1016/j.ntt.2011.10.007

Perron, B. E., Glass, J. E., Ahmedani, B. K., Vaughn, M. G., Roberts, D. E., & Wu, L. T. (2011). The prevalence and clinical significance of inhalant withdrawal symptoms among a national sample. *Substance Abuse and Rehabilitation, 2*, 69-76.

Perron, B. E., Howard, M. O., Vaughn, M. G., & Jarman, C. N. (2009). Inhalant withdrawal as a clinically significant feature of inhalant dependence disorder. *Med Hypotheses, 73*(6), 935-937. doi:10.1016/j.mehy.2009.06.036

Prajapati, R. P., Kalaria, M. V., Karkare, V. P., Parmar, S. K., & Sheth, N. R. (2011). Effect of methanolic extract of Lagenaria siceraria (Molina) Standley fruits on marble-burying behavior in mice: Implications for obsessive-compulsive disorder. *Pharmacognosy Res, 3*(1), 62-66. doi:10.4103/0974-8490.79118

Prediger, R. D., da Silva, G. E., Batista, L. C., Bittencourt, A. L., & Takahashi, R. N. (2006). Activation of adenosine A1 receptors reduces anxiety-like behavior during acute ethanol withdrawal (hangover) in mice. *Neuropsychopharmacology, 31*(10), 2210-2220. doi:10.1038/sj.npp.1301001

Ridenour, T. A., Bray, B. C., & Cottler, L. B. (2007). Reliability of use, abuse, and dependence of four types of inhalants in adolescents and young adults. *Drug Alcohol Depend, 91*(1), 40-49. doi:10.1016/j.drugalcdep.2007.05.004

Riegel, A. C., Ali, S. F., & French, E. D. (2003). Toluene-induced locomotor activity is blocked by 6-hydroxydopamine lesions of the nucleus accumbens and the mGluR2/3 agonist LY379268. *Neuropsychopharmacol, 28*(8), 1440-1447.

Riegel, A. C., & French, E. D. (2002). Abused inhalants and central reward pathways: electrophysiological and behavioral studies in the rat. *Ann N Y Acad Sci, 965*, 281-291.

Riegel, A. C., Zapata, A., Shippenberg, T. S., & French, E. D. (2007). The abused inhalant toluene increases dopamine release in the nucleus accumbens by directly stimulating ventral tegmental area neurons. *Neuropsychopharmacol, 32*(7), 1558-1569. doi:10.1038/sj.npp.1301273

Rivera-Garcia, M. T., Lopez-Rubalcava, C., & Cruz, S. L. (2015). Preclinical characterization of toluene as a non-classical hallucinogen drug in rats: participation of 5-HT, dopamine and

glutamate systems. *Psychopharmacology (Berl), 232*(20), 3797-3808. doi:10.1007/s00213-015-4041-8

Roberto, M., Cruz, M. T., Gilpin, N. W., Sabino, V., Schweitzer, P., Bajo, M., . . . Parsons, L. H. (2010). Corticotropin releasing factor-induced amygdala gamma-aminobutyric Acid release plays a key role in alcohol dependence. *Biol Psychiatry, 67*(9), 831-839. doi:10.1016/j.biopsych.2009.11.007

Rose, J. H., Karkhanis, A. N., Chen, R., Gioia, D., Lopez, M. F., Becker, H. C., . . . Jones, S. R. (2016). Supersensitive Kappa Opioid Receptors Promotes Ethanol Withdrawal-Related Behaviors and Reduce Dopamine Signaling in the Nucleus Accumbens. *Int J Neuropsychopharmacol, 19*(5). doi:10.1093/ijnp/pyv127

SAMHSA. (2012). *Results from the 2011 National Survey on Drug Use and Health: Summary of National Findings*. Rockville, MD: Substance Abuse and Mental Health Services Administration.

SAMHSA. (2014). *Results from the 2013 National Survey on Drug Use and Health: Summary of National Findings, NSDUH Series H-48, HHS Publication No. (SMA) 14-4863*. Rockville, MD: Substance Abuse and Mental Health Services Administration.

Schoenthaler, S. J., Blum, K., Braverman, E. R., Giordano, J., Thompson, B., Oscar-Berman, M., . . . Gold, M. S. (2015). NIDA-Drug Addiction Treatment Outcome Study (DATOS) Relapse as a Function of Spirituality/Religiosity. *J Reward Defic Syndr, 1*(1), 36-45. doi:10.17756/jrds.2015-007

Shah, R., Vankar, G. K., & Upadhyaya, H. (1999). Phenomeology of gasoline intoxication and withdrawal symptoms among adoelscents in India: a case series. *Am J Addict, 8*(3), 245-257.

Shelton, K. L., & Nicholson, K. L. (2013). Benzodiazepine-like discriminative stimulus effects of toluene vapor. *Eur J Pharmacol, 720*(1-3), 131-137. doi:10.1016/j.ejphar.2013.10.036

Shen, Y. C. (2007). Treatment of inhalant dependence with lamotrigine. *Prog Neuropsychopharmacol Biol Psychiatry, 31*(3), 769-771. doi:10.1016/j.pnpbp.2006.12.016

Siegel, J. T., Alvaro, E. M., Patel, N., & Crano, W. D. (2009). "...you would probably want to do it. Cause that's what made them popular": Exploring perceptions of inhalant utility among young adolescent nonusers and occasional users. *Subst Use Misuse, 44*(5), 597-615. doi:10.1080/10826080902809543

Stengard, K., Hoglund, G., & Ungerstedt, U. (1994). Extracellular dopamine levels within the striatum increase during inhalation exposure to toluene: a microdialysis study in awake, freely moving rats. *Toxicol Lett, 71*(3), 245-255.

Tegeris, J. S., & Balster, R. L. (1994). A comparison of the acute behavioral effects of alkylbenzenes using a functional observational battery in mice. *Fundam Appl Toxicol, 22*(2), 240-250.

Tenenbein, M., Casiro, O. G., Seshia, M. M., & Debooy, V. D. (1996). Neonatal withdrawal from maternal volatile substance abuse. *Arch Dis Child Fetal Neonatal Ed, 74*(3), F204-207.

Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L. A., & Paylor, R. (2009). Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology (Berl), 204*(2), 361-373. doi:10.1007/s00213-009-1466-y

Thurgood, S. L., McNeill, A., Clark-Carter, D., & Brose, L. S. (2016). A Systematic Review of Smoking Cessation Interventions for Adults in Substance Abuse Treatment or Recovery. *Nicotine Tob Res, 18*(5), 993-1001. doi:10.1093/ntr/ntv127

Tomaszycki, M. L., Aulerich, K. E., & Bowen, S. E. (2013). Repeated toluene exposure increases c-Fos in catecholaminergic cells of the nucleus accumbens shell. *Neurotoxicol Teratol, 40C*, 28-34. doi:10.1016/j.ntt.2013.09.001

Umathe, S., Bhutada, P., Dixit, P., & Shende, V. (2008). Increased marble-burying behavior in ethanol-withdrawal state: modulation by gonadotropin-releasing hormone agonist. *Eur J Pharmacol, 587*(1-3), 175-180. doi:10.1016/j.ejphar.2008.03.035

Valdez, G. R., Roberts, A. J., Chan, K., Davis, H., Brennan, M., Zorrilla, E. P., & Koob, G. F. (2002). Increased ethanol self-administration and anxiety-like behavior during acute ethanol withdrawal and protracted abstinence: regulation by corticotropin-releasing factor. *Alcohol Clin Exp Res, 26*(10), 1494-1501. doi:10.1097/01.ALC.0000033120.51856.F0

Van Skike, C. E., Diaz-Granados, J. L., & Matthews, D. B. (2015). Chronic intermittent ethanol exposure produces persistent anxiety in adolescent and adult rats. *Alcohol Clin Exp Res, 39*(2), 262-271. doi:10.1111/acer.12617

Verma, R., Balhara, Y. P., & Dhawan, A. (2011). Inhalant Abuse: An exploratory study. *Industrial Psychiatry Journal, 20*(12), 103-106.

Wang, Y., Liu, P. P., Li, L. Y., Zhang, H. M., & Li, T. (2011). Hypothermia reduces brain edema, spontaneous recurrent seizure attack, and learning memory deficits in the kainic acid treated rats. *CNS Neurosci Ther, 17*(5), 271-280. doi:10.1111/j.1755-5949.2010.00168.x

Wick, R., Gilbert, J. D., Felgate, P., & Byard, R. W. (2007). Inhalant deaths in South Australia: a 20-year retrospective autopsy study. *Am J Forensic Med Pathol, 28*(4), 319-322. doi:10.1097/PAF.0b013e31815b48b0

00000433-200712000-00009 [pii]

Williams, J. F., Storck, M., Abuse, A. A. o. P. C. o. S., & Health, A. A. o. P. C. o. N. A. C. (2007). Inhalant abuse. *Pediatrics, 119*(5), 1009-1017. doi:10.1542/peds.2007-0470

Williams, J. M., Stafford, D., & Steketee, J. D. (2005). Effects of repeated inhalation of toluene on ionotropic GABA A and glutamate receptor subunit levels in rat brain. *Neurochem Int, 46*(1), 1-10. doi:S0197-0186(04)00156-1 [pii]

10.1016/j.neuint.2004.07.006

Win-Shwe, T. T., & Fujimaki, H. (2012). Acute administration of toluene affects memory retention in novel object recognition test and memory function-related gene expression in mice. *J Appl Toxicol, 32*(4), 300-304. doi:10.1002/jat.1693

Wood, R. W., Coleman, J. B., Schuler, R., & Cox, C. (1984). Anticonvulsant and antipunishment effects of toluene. *J Pharmacol Exp Ther, 230*(2), 407-412.

ABSTRACT

A MOUSE MODEL OF TOLUENE ABSTINENCE-INDUCED PATHOLOGY

by

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Advisor: Dr. Scott Bowen

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The intentional misuse of volatile solvents is a persistent public health concern. Limited self-report data suggests that chronic inhalant abusers experience withdrawal symptoms including anxiety and seizure symptoms. However, these symptoms have never been explored in a preclinical model and are not considered part of the DSM-V criteria for an Inhalant Use Disorder. For this experiment, 76 young adult male Swiss Webster mice were exposed to either 5,000 ppm toluene vapor or air (0 ppm) for 24 consecutive hours beginning on postnatal day (PND) 30. Following the 24 hour exposure, mice were allowed to recover for 3 hours before behavioral testing began. In the 1st experiment, mice were tested for handling-induced seizure activity every hour for 6 hours (and again at 24 hours). As compared to controls, toluene-abstinent animals showed persistent clonic seizure activity throughout the 6 hour period. In the 2nd experiment, mice were given a single i.p. injection of pentylenetetrazol (PTZ; 42 or 48 mg/kg) to induce seizure activity. Mice were observed for 30 min and seizure activity was scored for severity using criteria adapted from the Functional Observational Battery. As compared to air controls, toluene-abstinent mice displayed a significant increase in seizure symptoms. In the 3rd experiment, previously exposed toluene mice were re-exposed to toluene vapor for 30 min following the three hour abstinence

period. Following toluene re-exposure, these mice were tested for seizure severity with 42 or 48 mg/kg PTZ. Toluene re-exposure significantly reduced the severity of the seizure response. Next, we examined anxiety-like behavior arising 24 hours following 24 hour toluene exposure. Mice were tested in the Elevated Plus Maze, Open Field Test, Light/Dark Box, and Marble Burying task at either 24 or 72 hours of abstinence. Mice 24, but not 72 hours abstinent from toluene displayed increased anxiety-like behavior. Taken together, these results suggest that toluene abstinence lowers seizure threshold in mice and increases anxiety and that toluene re-exposure ameliorates these effects. These findings provide support for clinical reports of a physical withdrawal syndrome from inhalants, which has implications for the successful diagnosis and subsequent treatment of Inhalant Use Disorders.

AUTOBIOGRAPHICAL STATEMENT

As an undergraduate, I was fortunate enough to work in the clinical substance abuse field, where I became passionate about behavioral pharmacology. During my undergraduate years, I worked with two different faculty members on two very distinct projects. With Dr. Dean Purcell I worked at developing a method of facial affect recognition to assess effects of top-down processing in the accurate recognition of emotions and threat detection. For Dr. Kanako Taku, I developed novel multivariate and latent variable models of positive life change following personal life trauma. I applied exploratory factor analysis and structural equation modelling techniques to large clinical datasets. Upon graduating, I was accepted into a behavioral neuroscience PhD program at Wayne State University. While working under the direction of Dr. Scott Bowen, I earned my Master's degree studying the *in-vivo* neurochemical and behavioral effects of exposure to toluene vapor. My previous experience from my undergraduate work and work in Dr. Bowen's lab has provided me with the skills and knowledge I need to successfully complete the proposed project. During the first three years of my graduate study at Wayne State University, I developed a novel dynamic exposure system that would allow for simultaneous measurement of activity and microdialysis sampling in a freely moving animal during volatile solvent exposure. Once developed, I utilized this system to explore the relationship between GABA and DA dynamics in the mouse caudate putamen following exposure to the volatile solvent toluene. I also used reverse-dialysis techniques to assess changes in cellular sensitization following toluene exposure.